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Endocrine-Disrupting Chemicals: Prepubertal Exposures and Effects on Sexual Maturation and Thyroid Function in the Male Rat. A Focus on the EDSTAC Recommendations

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ABSTRACT: Puberty in mammalian species is a period of rapid interactive endocrine and morphological changes. Therefore, it is not surprising that exposure to a variety of pharmaceutical and environmental compounds has been shown to dramatically alter pubertal development. This concern was recognized by the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) that acknowledged the need for the development and standardization of a protocol for the assessment of the impact of endocrine-disrupting compounds (EDC) in the pubertal male and recommended inclusion of an assay of this type as an alternative test in the EDSTAC tier one screen (EPA, 98). The pubertal male protocol was designed to detect alterations of pubertal development, thyroid function, and hypothalamic-pituitary-gonadal (HPG) system peripubertal maturation. In this protocol, intact 23-day-old weanling male rats are exposed to the test substance for 30 days during which pubertal indices are measured. After necropsy, reproductive and thyroid tissues are weighed and evaluated histologically and serum taken for hormone analysis. The purpose of this review was to examine the available literature on pubertal development in the male rat and evaluate the efficacy of the proposed protocol for identifying endocrine-disrupting chemicals. The existing data indicate that this assessment of puberty in the male rat is a simple and effective method to detect the EDC activity of pesticides and toxic substances.

KEY WORDS: thyroid function, hypothalamic-pituitary axis.

I. INTRODUCTION

Puberty in mammalian species is a period of rapid interactive endocrine and morphological changes. Therefore, it is not surprising that exposure to a variety of pharmaceutical and environmental compounds has been shown to alter the timing of pubertal development in animals. This concern was recognized by the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC), which

acknowledged the need for the development and standardization of a protocol for the assessment of the impact of endocrine-disrupting compounds (EDC) in the pubertal male and recommended inclusion of an assay of this type as an alternative test in the EDSTAC tier one screen (EPA, 98). The pubertal male protocol was designed to detect alterations of pubertal development, thyroid function, and hypothalamic–pituitary–gonadal (HPG) system peripubertal maturation. In this modified protocol (see Table 1), intact 23-day-old weanling male rats are exposed to the test substance for the 30 days during which pubertal indices are measured. After necropsy, reproductive and thyroid tissues are weighed and evaluated histologically and serum taken for hormone analysis.

The purpose of this review is to examine the available literature on pubertal development in the male rat and evaluate the efficacy of the proposed protocol for identifying endocrine-disrupting chemicals. To accomplish this, a discussion of the mechanisms that are known, or strongly suspected, to regulate the onset of puberty in the male rat will be presented. This discussion is followed by a review of the literature for those compounds known to alter male reproductive development and thyroid function. In this regard, our review focuses on those studies that report the same or similar exposure

TABLE 1
Research Protocol for Assessment of Pubertal Development and Thyroid Function in Immature (23- to 53-Day-Old) Male Rats

Purpose and applicability

The purpose of this protocol is to outline procedures to quantify the effects of environmental compounds on pubertal development and thyroid function in the intact juvenile/peripubertal male rat. This assay detects compounds that display antithryoid, estrogenic, androgenic, antiandrogenic [androgen receptor (AR) or steroid enzyme mediated] activity, or alters puberty via changes in follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin, growth hormone (GH) or hypothalamic function.

Required endpoints
- Growth (body weight)
- Age and weight at preputial separation
- Serum thyroxin (T4) and thyroid-stimulating hormone (TSH)
- Thyroid histology
- Seminal vesicle plus coagulating gland weight (with and without fluid)
- Ventral prostate weight
- Levator ani plus bulbocavernosus weight
- Epididymal and testis weights and histology

Optional measures
- Serum testosterone, estradiol, LH, prolactin and tri-iodothyronine (T3)
- Liver, kidney, adrenal and pituitary weights and histology
- *ex vivo* testis and pituitary hormone production
- Hypothalamic neurotransmitter levels

Abbreviations: ANOVA, analysis of variance; **AR,** androgen receptor; **CV,** coefficient of variation; **DA,** dopamine; **DDE,** 1,1-dichloro-2,2-*bis*(4-chlorophenyl)ethylene; **DHT,** dehydrotestosterone; **dp,** dorsal prostate; **EDSTAC,** Endocrine Disrupters Screening and Testing Advisory Committee; **EOP,** endogenous opioid peptide; **Epi,** epididymis; **FSH,** follicle-stimulating hormone; **GABA,** gamma amino butyric acid; **GD,** gestation day; **GH,** growth hormone; **GHRH,** growth hormone releasing hormone; **GnRH,** gonadotropin-releasing hormone; **H2,** histamine; **HCG,** human chorionic gonadotropin; **HPG axis,** hypothalamic-pituitary-gonadal axis; **HPTE,** 2,2-*bis*-(*p*-hydroxyphenyl)-1,1,1-trichloroethane; **HSD,** hydroxysteroid dehydrogenase; **IGF-1,** Insulin-like growth factor 1; **ip,** intraperitoneal; **LABC,** levitor ani/ bulbocavernosus; **LC,** Leydig cell; **LH,** luteinizing hormone; **Lhr,** luteinizing hormone receptor; **LP,** lateral prostate; **ME,** median eminence; **NE,** norepinephrine; **NMDA,** *N*-methyl-D-aspartate ; **NPY,** neuropeptide Y; **4-OHE2,** 4-hydroxyestradiol; **2-OHE2,** 2-hydroxyestradiol; **PND,** postnatal day; **PPS,** preputial separation; **PRL,** prolactin; **PTU,** propyl thiouracil; **5αR,** 5 alpha-reductase; **sc,** subcutaneous; **SE,** standard error; **SV,** seminal vesicle; **T,** testosterone; **T1S,** Tier 1 screen; **T3, 3,5,3′,** triiodothyronine; **T4,** thyroxine or 3,5,3′,5′-tetraiodothyronine; **TCDD,** dioxin or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; **TSH,** thyroid-stimulating hormone; **VP,** ventral prostate.

TABLE 1 (continued)
Research Protocol for Assessment of Pubertal Development and Thyroid Function in Immature (23- to 53-Day Old) Male Rats

A. General Conditions

Rats are housed in clear plastic cages (20 × 25 × 47 cm) with heat-treated (to eliminate resins that induce liver enzymes) laboratory-grade pine shavings as bedding. Animals are maintained on a complete and balanced laboratory diet and tap water ad libitum, in a room with a 14:10-h photoperiod (lights on at 0500 h, off at 1900 h), temperature of 20 to 24°C and a relative humidity of 40 to 50%. Reasonable variations of this portion of the protocol should be acceptable when documented and justified.

B. Animals-juvenile male rats

The study will use male Sprague-Dawley or Long-Evans hooded rats weaned on day 21. The litters may be derived from individually housed pregnant females that were bred in house matings or purchased from a supplier as "timed pregnant" on days 7 to 10 of gestation. Enough litters should be available to assure that a sufficient number of juvenile males are available to meet the requirement of 15 pups per treatment group. To maximize uniformity in growth rates, the litters are culled to eight to ten pups per dam at postnatal day 3 or 4, and body weight should be monitored on a weekly basis with any unthrifty litters or runted pups excluded from the study. On day 21, the pups are weighed to the nearest 0.1 g and weight ranked. A population of male rats that is as homogeneous as possible is selected for the study by eliminating the "outliers" (i.e., the largest and smallest of the pups with a range of 8 grams above and below the mean used as a guideline). Pups are then assigned so that treatment groups exhibit similar body weight means and variances. In this regard, one nuisance variable, i.e. body weight at weaning, is experimentally controlled. After assignment to treatment groups, similarly treated males can be housed 2 to 3 per cage

C. Experimental Design

The design is a randomized complete block (body weight at weaning is the blocking factor) design with 15 weanling male rats in each of two treatment groups. The treatment conditions are (1) vehicle treated, and (2) xenobiotic treated. If necessary, the study can be conducted in blocks rather than at one time. In this case, the blocks should contain all treatment conditions and balanced with respect to numbers of animals in each block (i.e., two blocks with two treatment conditions, with eight males/treatment/block). Varying dosage levels of the xenobiotic can be tested, although only one high dosage level at or just below the maximum tolerated dose (MTD) or limit dose is required.

D. Treatment

Treatments are administered daily by oral gavage beginning at 23 days of age and continuing through 53 days of age. The dose should be administered between 0700 and 0900 h using an 18-gauge gavage needle (1 inch length, with a 2.25 mm ball) and a 1 cc glass tuberculin syringe in a volume of 2.5 to 5.0 ml corn oil/kg body weight. Doses should be administered on a mg/kg body weight basis which are adjusted daily for weight changes. Body weight (nearest 0.1 g) and the volume of the dose administered (nearest 0.1 ml) are recorded daily.

E. Preputial Separation (PPS)

Males are examined daily for PPS. The appearance of partial and complete PPS, or a persistent thread of tissue between the glans and prepuce should all be recorded if and when they occur. In addition, the weight at complete PPS should be recorded.

F. Necropsy

The method of euthanasia on PND53 will depend on the endpoints desired. If pituitary hormone analyses are not included, the males may be killed with CO2 on the last day of treatment. If pituitary hormone analyses are to be included the males should be killed by decapitation, which is conducted in a room separate from the housing area and within 15 s of the animal's removal from the cage. Blood is collected, centrifuged, and stored in siliconized microcentrifuge tubes at –20°C for subsequent thyroxine (T4) and TSH measurements. At necropsy, the testes, the paired testicular, paired epididymal, liver, ventral prostate, seminal vesicle (with coagulating glands and fluid), levator ani plus bulbocavernosus muscles, to the nearest milligram and body weights (nearest 0.1 g) should be recorded. During necropsy care must be taken to remove mesenteric fat

TABLE 1 (continued)

from the seminal vesicles and coagulating gland so that the weight of these tissues may be recorded with and without fluid. The thyroid, testes, and epididymides are prepared for histological evaluation by placing them in Bouin's fixative for 24 h, after which they are rinsed and stored in 70% alcohol, until being embedded in paraffin, sectioned and stained with hematoxylin and eosin.

G. Statistical Analysis

All data (age at PPS, weight at PPS, body and organ weights at necropsy, and serum hormones) are analyzed using multivariate analysis of covariance with the body weight at weaning as the covariate. If the treatment x body weight interaction is not significant, then differences among treatment means may be tested using a two-tailed test. Combining all endpoints into a MANCOVA will assure that the issues involving multiple comparisons because of the number of endpoints examined will be properly addressed. If data display heterogeneity of variance, then appropriate data transformations or the use of nonparametric analysis should be employed. Often log transformation of serum hormone data is required because the variance is not proportional to the mean.

H. Data Summary

Tables providing data from individual animals should be provided in conjunction with a summary table providing the mean, standard error of the mean (SEM), and sample size for each treatment group. The mean, SEM, and CV values for the control data should be examined to determine if they meet acceptable QA criteria for consistency with normal values. Data presented should include age and weight at PPS, testicular, epididymal, ventral prostate, seminal vesicle (with coagulating glands and fluid), levator ani, liver and body weights at necropsy, body weight change from day 21 to necropsy and serum T4 and TSH. Data may be also be presented after covariance adjustment for body weight, but this should not replace presentation of the unadjusted data. A summary of any histological findings should be included.

parameters (i.e., PND 23-53). In the final section, we offer comment on the potential strengths and limitations of the protocol itself in light of the literature review.

II. PUBERTAL MATURATION IN THE MALE RAT

Puberty is the transitional period between the juvenile and adult state in which sexual maturation begins in the hypothalamic–pituitary–gonadal (HPG) system, leading to the development of secondary sex characteristics and fertility. The three primary controllers in the HPG axis are gonadotropin-releasing hormone (GnRH) neurosecretory neurons within the hypothalamic arcuate nucleus, the pituitary gonadotropes (which secrete luteinizing hormone and follicle stimulating hormone) and lactotropes (which secrete PRL), and the gonadotropin-responsive elements within the testes (Leydig and sertoli cells).

The basic mechanisms involved in the progression of sexual maturation are conserved across most species. Many of the endocrine-mediated events that are involved in this progression in the rat are similar to other mammalian species, including humans. For example, the control of GnRH, the release of gonadotropins from the pituitary, and the steroid positive and negative feedback are relatively consistent across mammalian species (Ojeda and Urbanski, 1994). However, there do appear to be several differences between the human and rat pubertal progression. For example, male rodents do not display a postnatal period of testicular quiescence analogous to that in the human juvenile period, but rather show initiation and progression of testicular development at a very early age (Ojeda and Urbanski, 1994). Postnatal sexual development in the male rat has been classified into four periods: (1) neonatal, postnatal day (PND) 1-7, (2) infantile (PND 8-21), (3) juvenile, (PND 22-35), and (4) peripubertal, (PND 36- 55 or 60), at which time the first mature spermatozoa appear in the vas deferens (Clegg, 1960; Ojeda et al., 1980). In the rat, testicular descent normally occurs after PND 15. The separation of the foreskin of the penis from the glans, preputial separation (PPS), is an early marker of the progression of puberty, which normally occurs between 40 and 50 days of age with

an average of 43 days, depending on the rat strain (Korenbrot et al., 1977). The first spermatozoa are observed in the lumen of the seminiferous tubules by 45 days of age (Clermont and Perey, 1957), and reach the vas deferens by 58 days (Clegg, 1960).

The mechanisms underlying the onset of puberty in the male rat are not as clearly defined as in the female (see accompanying review). Also, the onset of puberty in the male, within the HPG, is a more gradual progression when compared with the female (Ojeda and Urbanski, 1994). Unlike the female, the interrelationship within the HPG is already functional during late gestation, with the first few postnatal weeks representing a period of synchronization of the system (see Table 2 for ontogeny of some of the receptors and hormones involved in this synchronization). There is much agreement in the current literature that the primary events that set into motion the onset of male puberty originate within the central nervous system, with the testes playing a critical role. The subsequent testicular changes that take place during the juvenile and peripubertal periods, including the increased sensitivity of the testes to stimulation by gonadotropins, play pivotal roles in several aspects of adult function, including behavior, spermatogenesis, and maintenance of accessory sex organs. In addition to those changes within the HPG, other factors can affect the timing of puberty in the male rat, including nutritional status (leptin/body weight) and environmental (pineal gland/secretion of indole amines) influences.

III. HYPOTHALAMIC-PITUITARY AXIS

As mentioned above, there is general consensus that the central nervous system (CNS) is the primary initiator of the onset of puberty in the male rat. However, the exact sequence of change and mechanisms involved are yet to be elucidated. Key to this assumption are observations that include prepubertal changes in (1) GnRH neuronal shape, (2) GnRH release, and (3) the concentration of neurotransmitters and other trophic molecules within the hypothalamus that regulate GnRH release. To support further the role of the CNS as the primary site for the initiation of puberty, it has been shown that prior to puberty the male rat is fully capable of responding to GnRH with robust increases in LH and subsequently testosterone and the fact that hCG will fully stimulate steroidogenesis at these times (Cicero et al., 1986).

A. Gonadotropin-Releasing Hormone

GnRH is secreted in a pulsatile manner from the median eminence of the hypothalamus into the hypophyseal portal vessels, and, in turn, it stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. As puberty approaches, the GnRH neurons in the male rat undergo morphological changes. There is an increase in the proportion of cells with spiny-like processes relative to the smooth processes seen prior to puberty (Wray and Hoffman, 1986). This may reflect a puberty-related increase in the number of synapses on the cells, because it has been shown that spiny GnRH neurons have a greater number of endings (Jennes et al., 1985), which leads to an enhancement of the pubertal rise of gonadotropin secretion. This rise in gonadotropin secretion, in turn, stimulates the growth and maturation of the testes.

The pulsatile release of GnRH also shows a doubled pulse frequency between the infantile (15 days) and the peripubertal periods (50 days) in the male rat (Bourguignon et al., 1994). The initiator of this increased pulsatility has sometimes been termed the "GnRH-pulse generator", which signifies a network of neurons that are involved in the coordinated synchronous pattern in which GnRH cells discharge their products into the hypophyseal portal blood (Evans and Karsch, 1995). Several observations suggest that this increase in frequency prior to puberty may be due to a combination of changes that take place in neuronal inputs, with "activation of excitatory" or "deactivation of inhibitory" inputs to GnRH neurons (Ojeda and Urbanski, 1994). Among the numerous CNS cell types controlling GnRH neurons, there are three stimulatory and two inhibitory neurotransmitter systems that appear to be most predominant (Crowley et al., 1995). The excitatory neurotransmitter systems include norepinephrine (NE) (Ramirez et al., 1984), neuropeptide Y (NPY) (Crowley et al., 1995), and the excitatory amino acids (EAA) (Ojeda and Urbanski, 1994), whereas the inhibitory neuronal systems include γ-aminobutyric acid (GABA) (Mosotto and Negro-Vilar, 1987; Terasawa, E, 1995) and the endogenous

TABLE 2
Ontogeny of Receptors and Hormones in the Male Rat

Steroid or Protein	GD-18 to Birth	PND 1-15	PND 16-25	PND 26-35	PND36-45	PND46-55	PND56-65	PND66-90	Reference
GnRH pulsatility	+	+	+++	+++	+++	+++	Pulsatile	Pulsatile	(7)
GnRH receptor	gd-16-birth (7) +	+	+	+++	+++	+++	++	↓	(11)
LH receptor	gd-15 (3) +	+	++	++	+++	+++	+++	+++	(2)
FSH	+ (8,1)	+	+	+++	+++	++	++	++	(2,4)
FSH receptor semi-tubules	+	+++ pnd 10-15	+++	++	↓				(2)
Prolactin	+	+	+	+++	+++	↓			(8,9)
Inhibin B		+ pnd-3 ++ pnd10-15	+++	++	+				(17)
TSH (plasma)	+++ (14)	+ to +++ (14, 16)	++	+++	+++	++	++	++	(10, 16)
T3		+ to ++	+++	+++	++	++	+		(13,14,15)
T4		+ to ++	+++	+++	+++	++	++		(13,14)
AR testes	+	++ (14)	+	++	++	+++	+++	+++	(12)

Note: ↓ = decreased to adult levels, + = low level, ++ = moderate level, and +++ = high level. Blank cells represent undetermined levels. (1) Chowdhury and Steinberger, 1976; (2) Ketelslegers et al., 1978; (3) Picon and Gangnerau, 1980; (4) Matsumoto et al., 1986; (5) Aubert et al., 1985; (6) Ojeda and Urbanski, 1994; (7) Bourguignon and Franchimon, 1984; (8) Dohler and Wuttke, 1975; (9) Beucu and Libertun, 1982; (10) Simpkins et al., 1976; (11) Dalkin et al., 1981; (12) Buzek et al., 1988; (13) Martin et al., 1982; (14) Fukuda and Greer, 1978; (15) Sharpe et al., 1999.

opioid peptides (EOPs) (Bhanot and Wilkinson, 1983).

Although it has been proposed that there may be a "loss of central restraint" or deactivation of inhibitory input involved in the prepubertal increase in GnRH activity, such as the removal of opiate or GABAergic input, support for this hypothesis is limited (Nazian, 1992; Ojeda and Urbanski, 1994). However, it is has been shown that GABA stimulates GnRH release in the immature male rat and is inhibitory in the adult male (Feleder et al., 1996), a phenomenon also observed in the female. Therefore, GABA may participate in pubertal development by playing a dual role in the neuronal control of GnRH release.

Similarly, the endogenous opioids appear to be inhibitory to GnRH in the adult male but without effect in the immature animal, a change in function that has been attributed to maturation of the opioid system following the peripubertal testosterone rise (Nazian, 1992). Naloxone, an opioid antagonist, has an effect on LH that is coincident with the onset of puberty (Bhanot and Wilkinson, 1983; Blank et al., 1979; Cicero et al., 1991), which implies that the EOPs that tonically inhibit LH secretion in adults are inactive before puberty (Cicero et al., 1993). Thus, like GABA-nergic action, it appears that marked differences are present before and after sexual maturation in the response of the HPG to opiate agonists and antagonists.

In the male, substantial evidence supports the hypothesis that a "central drive" or activation of excitatory inputs, such as activation of the excitatory amino acids (glutamate and aspartate) is involved in the initiation of puberty in the male. Glutamate and aspartate operate via the glutamate receptors of the *N*-methyl-D-aspartate (NMDA) subtype (Bourguignon et al., 1994; Bourguignon et al., 1990; Goldsmith et al., 1994). The activation of the NMDA receptors increases before the onset of puberty in a gonadal-independent manner (Bourguignon et al., 1992; Bourguignon et al., 1990). Therefore, the initiation of puberty could involve a facilitory effect of an EAA mediated through NMDA receptors. There is also evidence that glutaminase activity (which converts glutamine to glutamate) has increased activity at the onset of puberty, which parallels the increased GnRH pulsatility and secretion (Bourguignon et al., 1994). In addition to the NMDA receptors, there is some evidence that those EAAs that operate via the kainate receptor subtype may also be involved in the regulation of GnRH activity prior to puberty in the male rat (Pinilla et al., 1998).

It is well known that the neurotransmitters norepinephrine (NE) and dopamine (DA) modulate the release of GnRH. NE primarily has a facilitory effect on the release of GnRH, whereas the effects of DA are more complex, with both stimulatory and inhibitory effects (Barraclough and Wise, 1982). NE stimulates the release of GnRH by binding to the alpha-noradrenergic receptor (i.e., LH release can be effectively decreased by alpha-adrenergic antagonists or by drugs that depress NE synthesis). DA is primarily considered to be inhibitory to gonadotropin release, because an inverse relationship is observed between DA turnover in the median eminence and plasma LH levels (Naumenko and Serova, 1976). Changes in the activity of these neurotransmitters take place during the pubertal onset period. For example, Matsumoto et al. 1986 showed an increase in hypothalamic GnRH, NE and DA concentration, and NE turnover rate (i.e., neuronal activity) between 36 and 56 days of age. This change does not appear to be influenced by gonadal hormones (Choi and Kellogg, 1992), which is further evidence for a central onset of puberty in the male. Raum and Swerdloff, 1986) found that alpha-methyl-*p*-tyrosine (AMPT), a tyrosine hydroxylase inhibitor, increased serum LH and testosterone (T) on PND 15 and 23, but had the opposite effect on PND 38, 56, and 72. This suggested that the action of these neuronal systems changed during puberty from an inhibitory to stimulatory action.

In female rats, it has been proposed recently that glial cells also participate in the central control of mammalian puberty (Ojeda and Ma, 1998). Astrocytes affect GnRH neuronal function via cell-cell signaling mechanisms involving several growth factors acting via receptors endowed with tyrosine kinase activity. It has been shown that two members of the epidermal growth factor/transforming growth factor alpha (EGF/TGFα) family, and their respective receptors play a role in the glial-neuronal interactive process that regulates GnRH secretion. TGFα and neuregulin are produced in hypothalamic astrocytes and are able to stimulate GnRH release indirectly via activation of their receptors located on the astrocytes and not on the

neurons. Activation of the EGF receptor by TGFα, or the erbB2/erbB4 receptor complex by neuregulin, leads to glial release of prostaglandin E2, which in turn acts on the GnRH neurons to stimulate release. In the female, a central blockade of these two receptors delays puberty and focal overexpression of TGFα advances it (Ojeda and Ma, 1998). Therefore, these growth factors are considered to be components of the central mechanism controlling the initiation of puberty in females.

Another growth factor, insulin-like growth factor 1 (IGF-1), has also been shown to affect GnRH release in the median eminence, where the highest density of IGF-I receptors is located (Copeland et al., 1990; Hiney et al., 1991). In primary cultures of male rat anterior pituitary cells, IGF-I induced a dose-dependent release of LH in response to GnRH (Soldani et al., 1995). Plasma levels of IGF-1 change during the onset of puberty in both rodents and primates (Handelsman et al., 1987). The IGF-1 levels involved in the changes in GnRH secretion during puberty do not appear to be from a peripheral source such as the gonadal secretion of IGF-I (Ojeda and Urbanski, 1994), but from a hypothalamic source (Pazos et al., 1999). For example, the administration of IGF-I antibodies by intraventricular infusion to 28-day-old male rats resulted in a reduction in testicular weight and serum testosterone with a subsequent delay in pubertal development (Pazos et al., 1999).

A biopeptide, NPY, is also involved in the regulation of GnRH release in the adult through stimulation of the catecholaminergic input to the GnRH neurons. However, during development the content of NPY in the ME of males changes with age becoming maximum at PND 30 and then decreasing during sexual maturation (Corder et al., 1992). Increased NPY was also observed in male rats with delayed sexual maturation induced by injections of melatonin. These males had decreased pituitary GnRH receptor content and decreased testosterone secretion during treatment. This finding suggests an inhibitory maturational signal for NPY during puberty in the male, which has also been observed coincident with food restriction during sexual maturation (Gruaz et al., 1993).

GnRH receptors are already present in the male rat's pituitary by GD 16 (Aubert et al., 1985) and then increase during pubertal development along with hypothalamic receptor content (Table 2). The earliest measurement of serum LH and FSH occurred at GD15 for LH and GD19 for FSH (Ojeda and Urbanski, 1994), suggesting that the pituitary is responsive to GnRH at this stage in development. Postnatally, there is an increase in pituitary GnRH receptors and content of LH at about 30 days of age, which declines to adult levels by 60 to 80 days of age (Dalkin et al., 1981) (Table 2). This increase may be due to the rising testosterone levels, because it has been shown that testosterone can potentiate the pituitary response to GnRH in immature but not adult animals (Nazian and Mahesh, 1979).

B. Luteinizing Hormone

It is well known that LH stimulates testosterone secretion by a direct action on the Leydig cells. There has been considerable controversy over whether LH secretion increases as puberty approaches, with some laboratories reporting an increase beginning around PND 36 to 45 (i.e., Dohler and Wuttke, 1974; Ketelslegers et al., 1978; Lee et al., 1975). However, many more studies have found no alterations over the course of puberty in the male (i.e., Chiappa and Fink, 1977; Kamberi et al., 1980; Matsumoto et al., 1986; Ojeda and Urbanski, 1994). These inconsistencies have been suggested to be due to the pulsatile manner in which LH is released, even prior to and during puberty (Kimura and Kawakami, 1982), which can cause increased variability. There is, however, an increase in pituitary content of LH (peak) at about 30 days of age, which reaches adult levels by 60 to 80 days of age (Debeljuk et al., 1972; Duncan et al., 1983) (Table 2). In addition, there is a maximum release of LH in response to GnRH between 35 and 45 days of age, which is likely due to the rising testosterone levels, because, as previously mentioned, testosterone can potentiate the pituitary response to GnRH in immature males (Nazian and Mahesh, 1979). Although only subtle changes in serum LH are observed during puberty, there is an increased sensitivity of the testes to LH prior to puberty due to other hormonal influences that facilitate an upregulation of LH receptors (Kamberi et al., 1980; Odell et al., 1973; Vihko et al., 1991). LH receptors appear on the fetal testes around GD 15 and show a continuous increase in concentration

and total number with advancing age and testis growth. The major rise in receptor concentration occurs between PND 15 and 38, in parallel with the rise in plasma FSH and increased testicular growth (Ketelslegers et al., 1978) (Table 2).

C. Follicle-Stimulating Hormone

In contrast to the controversy over alterations in LH levels during puberty, there is agreement that FSH secretion increases prior to puberty in the male rat and that this hormone plays a vital role in the onset of puberty. FSH secretion rises during early postnatal life, reaches a maximum between 30 and 40 days of age, and then falls gradually as testosterone secretion increases, to remain low throughout adulthood (i.e., Ketelslegers et al., 1978; Matsumoto et al., 1986; Negro-Vilar et al., 1973a; Swerdloff and Walsh, 1973) (Table 2). A maximum release of FSH in response to GnRH is observed between PND 25 and 35 (Dullaart, 1977). It is possible that the hormone inhibin, which is secreted by the seminiferous tubules and inhibits FSH release, plays a role in the increase in serum FSH during puberty. There is an inverse relationship between inhibin levels and serum FSH, with an increase in inhibin from PND 3 to 20 and a decrease at PND 25 (Saito, 1995; Sharpe et al., 1999) (Table 2).

FSH binds to the Sertoli cells within the seminiferous tubules to facilitate spermatogenesis at puberty (Steinberger, 1976), and FSH has also been shown to enhance the production of steroid biosynthetic enzymes (Murono and Payne, 1979). The concentration of FSH receptors within the seminiferous tubules reaches a peak between PND10 and 15 and then falls to a constant level by PND 25 (Ketelslegers et al., 1978) (Table 2). As mentioned, FSH also increases LH receptors in the testis, a phenonmenon important for the increased production of testosterone during puberty (Odell et al., 1973). These observations demonstrate that the early development of these testicular FSH receptors, followed by the increased serum FSH levels, promotes testicular growth and the formation of LH receptors (Huhtaniemi et al., 1982; Odell and Swerdloff, 1976), which may occur through paracrine mechanisms (Vihko et al., 1991). Testicular growth parallels the FSH increase (Ojeda and Ramirez, 1972) and as the testes develop, serum FSH levels fall, even though mean LH levels increase slightly or remain relatively unchanged (Sheth et al., 1980). The fall in FSH that occurs following 40 days of age cannot be explained by alterations in the concentrations of inhibin or inhibin receptors because inhibin levels and inhibin binding sites on the pituitary gland continue to decrease with age (Sheth et al., 1980).

D. Prolactin

Serum prolactin levels are low until PND 20 and then rise significantly between 25 and 40 days of age in the rat (Becu and Libertun, 1982; Dohler and Wuttke, 1975; McCann et al., 1974; Piacsek and Goodspeed, 1978) (Table 2), with maximum prolactin levels coinciding with the start of accelerated growth in the prostate and seminal vesicle glands. It is well known that dopamine (DA) and thyrotropin-releasing hormone (TRH) can inhibit and stimulate the release of prolactin from the pituitary in the adult animal, respectively. Dopamine reaches the anterior pituitary through the hypophyseal portal vessels, and, as mentioned, plays a major role in prolactin inhibition. The gradual increase in prolactin levels during the third week of life is not related to a decrease in dopminergic inhibition but to an increase in the efficiency of prolactin-releasing factors such as estrogen, serotonin, and opiates (Becu-Villalobos et al., 1992). However, the responsiveness of the pituitary to the PRL-inhibiting effects of DA does increase during sexual maturation, beginning on PND21 (Karanth et al., 1987). For example, in the immature 25- to 30-day-old male, i.v. infusion of 160 μg of dopamine was necessary to inhibit PRL at 15 and 30 min, while at 50 to 55 days 40 μg was effective, and at 75 to 80 days only 10 μg was needed to inhibit PRL release (Nazian, 1983). In addition, the activity of tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (enzymes involved in dopamine synthesis), as well as the neonatal content of dopamine, increases with age (Hedner and Lundborg, 1981; Lamprecht and Coyle, 1972). In the median eminence, TH activity markedly increases from PND 10 to 20 and remains elevated through PND39. About this time dopamine receptor antagonists, such as pimozide and haloperidol, are able to affect prolactin release (Becu

de Villalobos et al., 1984). There also appears to be a peptidic nondopaminergic prolactin-inhibiting factor that disappears as the males mature (Laudon et al., 1989). It has been suggested that this factor may be gonadotropin-releasing hormone-associated peptide (GAP) (Becu-Villalobos et al., 1992).

Other changes that may be involved in the pubertal rise of prolactin secretion around the third postnatal week include changes in serotonin and opioid activity. For example, from PND 20 onward males are more sensitive to the prolactin-releasing effect of serotonin (Becu and Libertun, 1982). This has also been reported by Bero et al., 1987, who showed that a 5-HT receptor agonist and *p*-chloroamphetamine (an agent that stimulates serotonin release) stimulated prolactin release beginning on PND 15. The opioid control of prolactin during this period follows a similar pattern. For example, morphine increases plasma prolactin levels in 13- and 36-day-old rats, as well as adult males (Johnston and Negro-Vilar, 1986). However, naloxone is only able to lower prolactin in peripubertal males (Blank et al., 1980), with no effects prior to PND 36. This observation suggests, as previously mentioned, that the opioid regulation of prolactin occurs between PND 13 and 36.

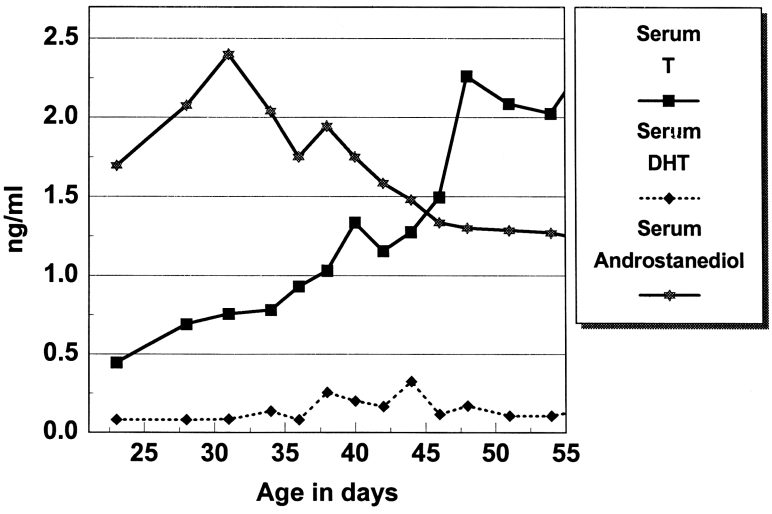
Histamine (H2) has also been shown to increase prolactin release during the third postnatal week. However, administration of cimetidine, an H2 antagonist of histamine, significantly increases serum prolactin beginning on PND 20 (Becu and Libertun, 1983).

Thus, it appears that the increases in serum prolactin about the third postnatal week require a maturation of the hypothalamic neurons at that time. Interestingly, it has been suggested that morphologic changes may account for the changes in prolactin regulation, because the number of axodendritic and spine synapse formations in the arcuate nucleus increase two- to threefold between PND 10 and 45 (Matsumoto and Arai, 1981).

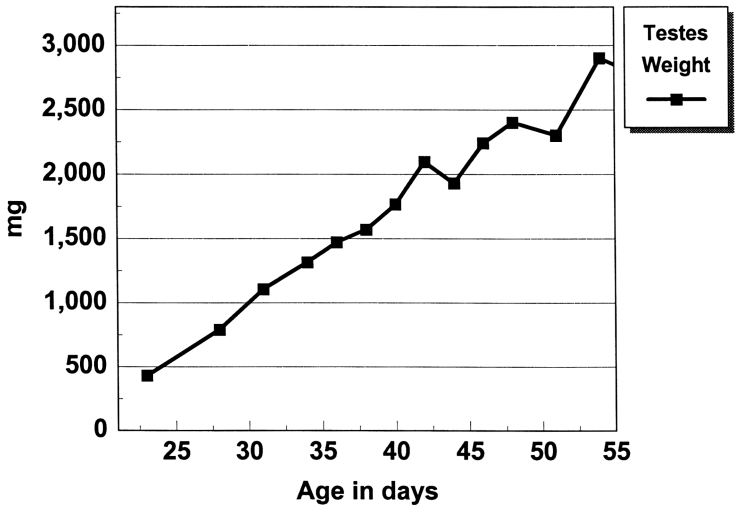
1. Effects of PRL on Testes and Sex Accessory Tissues

The increasing levels of serum prolactin prior to and during puberty have been suggested to be involved in testicular and accessory organ development and may facilitate the actions of luteinizing hormone, FSH, and testosterone on male sexual organs (Becu and Libertun, 1982; Negro-Vilar et al., 1973a). The initiation of the rapid growth of the testes at PND25 (e.g., Figure 1d) has been correlated with elevations of FSH and PRL. A pituitary graft under the kidney capsule, which would allow increased prolactin secretion free of dopaminergic inhibition, resulted in early preputial separation (Aguilar et al., 1988). It is believed that increases in prolactin cause an advancement in sexual maturation by synergizing with the action of LH and testosterone on male sex organs (de Jong and van der Schoot, 1979; Holland and Lee, 1980). This has been shown in the adult, with prolactin modifying androgen uptake in the prostate (Holland and Lee, 1980). Administration of a combination of prolactin and testosterone to castrated rats resulted in an increase in the weights of the sex accessory tissues that was greater than treatment with testosterone alone (Keenan and Thomas, 1975).

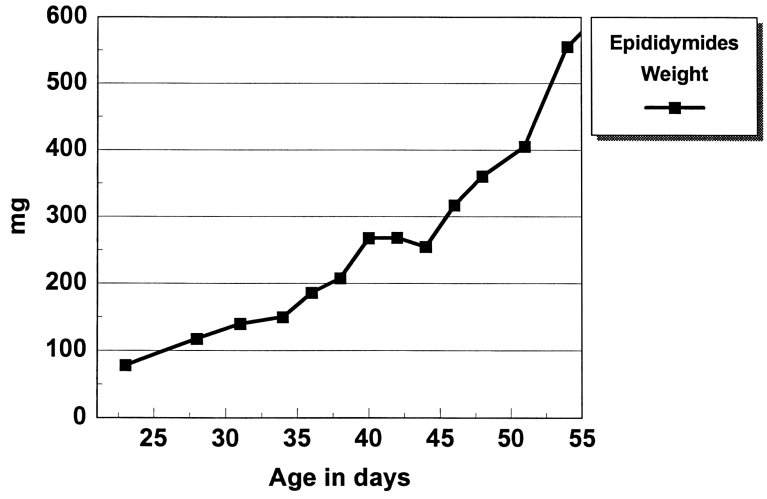
Prolactin receptors are also present in the testicular interstitial cells (Charreau et al., 1977), and prolactin has been shown to enhance testicular responsiveness to LH by increasing the number of LH receptors on the testes (Hafiez et al., 1971; Johnson and Brown, 1974; Kamberi et al., 1980; Kolena and Sebokova, 1983; McNeilly et al., 1979; Purvis et al., 1979; Zipf et al., 1978). The reverse is also true: at 30 days and later, treatment with the dopamine receptor agonist bromocriptine will suppress prolactin secretion and reduce LH binding to receptors in Leydig cells (Aragona et al., 1977). Bromocriptine treatment halves the number of LH receptors per million Leydig cells and reduces the *in vitro* steroid response of these cells to hCG (Purvis et al., 1979). In addition to having a direct stimulatory effect on the number of LH receptors on rat Leydig cells, prolactin also appears to act synergistically with LH to stimulate the quantity of androgen produced by the Leydig cells in response to hCG *in vitro* and to increase the sensitivity of the hCG dose-response. Therefore, it appears that prolactin may play a key role in maintaining the functional integrity of rat Leydig cells. Because of the effect of prolactin on LH responsiveness of the Leydig cells, a dose of naloxone (an opioid antagonist that decreases prolactin levels) peripubertally (PND 51-58) results in decreased serum androgen (Maric et al., 1987).


2.5
2.0
1.5
1.0
0.5
0.0
ng/ml
25 30 35 40 45 50 55
Age in days
Serum T
Serum DHT
Serum Androstanediol

(A)


3,000
2,500
2,000
1,500
1,000
500
0
mg
25 30 35 40 45 50 55
Age in days
Testes Weight

(B)


600
500
400
300
200
100
0
mg
25 30 35 40 45 50 55
Age in days
Epididymides Weight

(C)

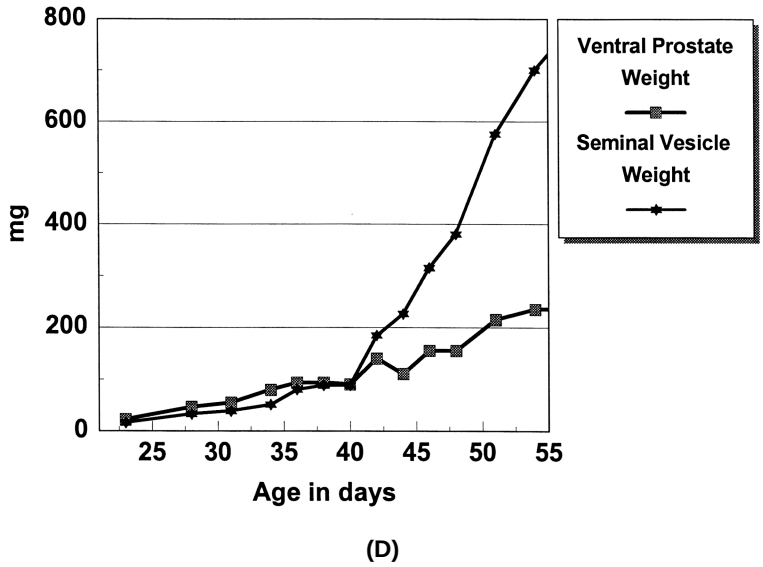


FIGURE 1. Ontogeny of circulating androgen levels, testes weight, and androgen-dependent tissue weights from PND-20 to PND-55; (A) serum testosterone, DHT and 3α-androstanediol measured by RIA as ng/ml, (B) paired testes weight measured in mg, (C) epididymides weight measured in mg, and (D) ventral prostate and seminal vesicle weights measured in mg. Data from Monosson et al., 1999.

It has also been suggested that prolactin may play a direct role in the maintenance of normal testicular steroidogenesis (Huhtaniemi et al., 1984). Prolactin has been shown to suppress the activity of 5α-reductase (which catalyzes the conversion of T to dihydrotestosterone or DHT) in the developing rat brain (Martini et al., 1978) and the adrenal cortex (Witorsch and Kitay, 1972).

Also, the growth of the sex accessory tissues can be associated with the secondary rise in prolactin beginning at 50 days of age (Negro-Vilar et al., 1973b). Other studies (Krall and Spring-Mills, 1982; Negro-Vilar et al., 1977) have reported that prolactin can stimulate growth of the ventral and dorsolateral prostate and increase seminal vesicle weights in castrated immature (21-day-old) male rats and also reverse the stromal proliferation and epithelial degeneration that normally follows prepubertal castration.

Specific binding sites for prolactin have been detected in membrane preparations from the testis, epididymis, seminal vesicles, and prostate of immature male rats (Aragona and Friesen, 1975). Specific binding sites for prolactin in the testis increased slightly from PND 20 to 70 in the rat. However, the highest specific binding was found in the prostate of 20-day-old pups. By comparison, the prostate of 270-day-old males had 10% as much specific binding. Specific binding of PRL in the Leydig cell was highest on PND 45 (Aragona et al., 1977), which correlates with the increased serum concentration of this hormone at this age. Thus, prolactin could be more effective in promoting prostate growth in young than in adult rats. It is interesting to note in the context of this paper that estrogen or castration lower prolactin binding to prostatic membranes (Aragona and Friesen, 1975).

E. Growth Hormone

Although exogenously administered growth hormone (GH) has been shown to increase the release of LH and testicular responsiveness to LH during sexual development (Kamberi et al., 1980), testicular endocrine function is normal in GH-deficient rats (Bartlett et al., 1990). GH-deficient rats also show normal spermatogenic function during pubertal development (Bartlett et al., 1990), suggesting that GH plays no role in spermatogenic function during puberty and adult life. However, a reduction in testicular size may reflect a reduced Sertoli cell population, suggesting that GH may be of importance in prepubertal testicular development (Bartlett et al., 1990).

GH is regulated by two hypothalamic peptides, somatostatin (growth hormone release inhibiting factor) and growth hormone releasing factor (GHRH), with somatostatin being the dominant controlling factor in the rat (Wehrenberg et al., 1982). The serotonergic and endogenous opiate systems are involved in regulation of GH secretion in rats and these systems are functional within the first postnatal week; however, the noradrenergic mechanism that triggers the surges of GH in the adult does not mature until after puberty (Kuhn and Schanberg, 1981). Hypothalamic somatostatin levels rise neonatally and peak around PND 28, followed by a decline toward puberty (Walker et al., 1977). In early puberty, between PND 33 and 40, rat GH pulse amplitude increases more than tenfold in both sexes. By late puberty, between PND 41 and 54, pulse amplitude further increases twofold in both sexes (Gabriel et al., 1992). Pituitary GH rises throughout the neonatal period to peak on PND 16 and peaks again by PND 60.

GH secretion is influenced by multiple factors, including gonadal steroids and IGF-I. There exists a synergistic effect of both GH and the sex steroids on growth during the normal pubertal process, with an androgen-mediated enhancement of both hypothalamic GRF release and pituitary GH secretion (Wehrenberg and Giustina, 1992). It appears that a low dose of estrogen and a high dose of testosterone are able to augment GH release, in part, at the level of the pituitary (Copeland et al., 1990). IGF-I, however, appears to inhibit GH at the level of the pituitary (Copeland et al., 1990; Yamashita and Melmed, 1986), and increase hypothalamic somatostatin release inhibiting factor production (Berelowitz et al., 1981). There also appears to be an effect of GH on IGF-I levels, because in GH-deficient dwarf rats, sex hormone levels appear normal, but there is no rise in IGF-1 at puberty as normally seen (Bartlett et al., 1990). This low IGF-1 was also demonstrated by Arsenijevic (Arsenijevic et al., 1989) when rats were immunized against GHRH.

F. Feedback Regulation in the HPG Axis

After birth, testosterone secretion and the hypothalamic-pituitary axis are regulated by negative feedback, which has been repeatedly demonstrated by surgical intervention (e.g., Nazian and Mahesh, 1980). There is a higher threshold for the gonadotropin/gonadal steroid feedback mechanism in the adult male (Gupta et al., 1975; Nazian and Mahesh, 1980) when compared with the immature male, making the immature male more sensitive to the feedback of testosterone. As this feedback sensitivity decreases, the hypothalamic-pituitary unit becomes more effective at stimulating testicular development, because there is less inhibition of gonadotropins by testosterone. It has also been reported that there is a decrease in hypothalamic aromatase and 5α-reductase activity during the peripubertal-adulthood transitional period (Lephart and Ojeda, 1990), suggesting that reduced hypothalamic metabolism of testosterone to its active metabolites estradiol and dihydrotestosterone may represent a factor underlying the peripubertal decrease in the sensitivity to gonadal steroid feedback that accompanies completion of sexual maturity.

Testosterone levels rise gradually from PND 20 to 40, and abruptly double by PND 50 (Matsumoto et al., 1986; Monosson et al., 1999) (e.g., Figure 1a). An increased turnover rate in hypothalamic GnRH, NE, and DA preceeds this increase in testosterone, rising between 36 and 56 days of age. At 56 days of age, there is also a demonstrable decrease in the hypothalamic-pituitary sensitivity to T's negative feedback when compared with 36 days of age. These observations have led to the "gonadostat theory" for the onset of puberty, where in a decrease in hypothalamic-pituitary sensivity to testosterone's negative feedback is postulated to allow intensive testicular production of testosterone. These neuroendocrine changes occur first, prior to the any noticeable change in circulating testosterone levels (Monosson et al., 1999).

It has also been shown that the testes secrete gonadal peptides, including IGF-I (Grizard, 1994), which suggests that the previously mentioned pubertal increases in plasma IGF-I levels might be attributed to either direct gonadal secretion of IGF-I or to indirect effects of gonadal steroids on the hypothalamus, pituitary, or liver. However, it has been reported that the pubertal IGF-1 increase in the rat is not due to direct gonadal secretion of IGF-I. Testosterone does not stimulate IGF-I gene expression in the liver, or does it increase IGF-I serum levels (Phillip et al., 1992).

IV. MODULATORY INFLUENCES ON PUBERTY ONSET

A. Growth/Leptin

Nutritional status and body weight of mammals have been well recognized as having effects on reproduction and puberty, which are suggested to reflect the metabolic signals in the brain that serve as indices of the metabolic state. It has also long been suggested that a low body weight may contribute to a delay in puberty (Kennedy and Mitra, 1963), with food restriction resulting in a delay in pubertal onset and refeeding reversing the delay (Kennedy and Mitra, 1963). However, other studies indicate that other factors, such as body fat and and rate of body weight growth, are more important than the attainment of a certain body weight (Glass et al., 1987). These investigators used weight-matched animals with varying patterns of body weight growth to examine their theory of a "critical body weight" requirement for pubertal progression in male rats. They found that the degree of sexual maturation correlated inversely with body fatness (i.e., leaner animals were more sexually mature) and rate of body weight growth, but correlated directly with body length. Still others hypothesize that a certain percentage of body protein is more likely to be a metabolic signal for puberty (Wilen and Naftolin, 1978). The idea that metabolic alterations associated with weight loss or decrease in growth rate are inhibitory to the reproductive system may be related to substances in the body that can alter the release of GnRH such as insulin, amino acids necessary for precursors of neurotransmitter synthesis, and essential fatty acids (Ojeda and Urbanski, 1994).

In addition to body weight, other signals of nutritional status appear to be involved in the reproductive status of the animal. Leptin is a protein hormone and product of the "obese gene" that has been implicated in the control of food intake and body weight (Zhang et al., 1994). It is synthesized in and secreted by fat cells in response to improved metabolic status, and it has the effect of increasing general metabolic rate and activity levels while decreasing appetite (Campfield et al., 1995). Leptin receptors are present in the brain (Cheung et al., 1997) and have been implicated as a link between nutrition and fertility (Barash et al., 1996). For example, mice with a mutation in the gene encoding leptin are infertile (Swerdloff et al., 1978).

Also, leptin decreases NPY, galanin (GAL), melanin-concentrating hormone (MCH), and propiomelanocortin (POMC) gene expression in the brain and increases the gene expression of neurotensin (NT) (Sahu, 1998). These neuropeptidergic systems play important roles in feeding and energy homeostasis.

Some toxicants administered during puberty can result in decreases or increases in body weight. For example, in addition to its effects on central and peripheral reproductive tissue, estrogen also has a direct effect on the CNS regions that regulate appetite by suppressing food intake (Reynolds and Bryson, 1974). Estrogenic exposures during puberty have also been shown to delay pubertal progression. Thus, exposure to doses of 100 or 400 mg/kg of methoxychlor beginning on PND 21 results in a significant decrease in body weight and a delay in PPS (Gray et al., 1988). Whether such a delay in puberty is due to estrogen's effects on reproductive target tissues, including the CNS, a suppression of gonadotropins or its effects on those CNS areas controlling the regulation of body weight or through both actions remains to be determined. The effects on body weight regulation appear to be at a low threshold dose of estrogen (Wade, 1972), which would make it hard to discern the mechanism of the delay. In the female, estrogen administered prior to puberty results in an advance of puberty, despite the decrease in body weight. In this regard, the female pubertal assay proves to be more sensitive to screen for estrogenic compounds. Other compounds administered peripubertally that resulted in a decreased weight include tetrahydrocannabinol (THC) and propyl thiouracil (PTU) (Table 3).

B. The Adrenal Gland

It has been suggested that the adrenal gland plays a role in puberty onset; however, most studies to date have been done in the female because it is more difficult to alter male pubertal onset. Adrenalectomy can delay puberty in several ways, including the direct result of the decreased availability

TABLE 3
Effects of Prepubertal Administration of Pharmacological and Environmental Chemicals on Pubertal Endpoints

Treatment	Dose (duration and route)	Strain	Effect	Putative Mechanism	Ref.
GnRH Modulators					
GnRH antagonist (Org 30039)	2mg/kg (d16-20, sc,2/d)	SD	delays PPS 1.4 d	GnRH antag.	Kolho et al.,1988
GnRH-Antagonist	d6-48, 5 mg/kg every 3d,sc	Wistar	delay PPS 40d, ↑sFSH, ↑testis wt., no△ LH or T (in the adult)		Van den Dungen et al., 1989
GnRH-Antagonist	d22-31, 5 mg/kg every 3d, sc	Wistar	delay PPS 13 d		Van den Dungen et al., 1989
GnRH-Antagonist	d23-d32, minipump 35ug/h/kg	SD	↓sLH, sFSH &sT, T,sv,vp wt.,		Huhtaniemi et al., 1984
GnRH antiserum	.8ml/kg/day d23-d32	SD	↓sLH, sFSH ↑pLH,↓PRL ↓T,sv,vp wt.	**Blocks GnRH, binding**	Huhtaniemi et al., 1984
GnRH	1,2.5,5.0 ug d28-41		sac d62 ↓sT, sex access, testes wt. delay PPS (1 wk) ↑5αR,↑sFSH (5.0)	Chronic GnRH down regulates pit. GnRH recept.	Wisner et al., 1983
α-Methyl-ρ-tyrosine (or AMPT)	250 mg/kg, ip, d15 and 23	Wistar	↑sLH and sT	TH inhibitor	Raum and Swerdloff, 1986
	d38, 56 and 72	Wistar	↓sLH and T	TH inhibitor	Raum and Swerdloff, 1986

Diazepam	d38		Inhib. sPRL	Benzodiazepam GABA	Lacau de Mengido et al., 1989
NMDA	d20 or d30, 15mg/kg, i.p.	Wistar	↑sLH &FSH	stim. gonadotropins	Aguilar et al., 1996
Kainic acid	2.5mg/kg d20 or d30	Wistar	↑sLH &FSH	stim. gonadotropins	Aguilar et al., 1996
Opioid Modulators					
Morphine	15 mg/kg, sc ,d25, 30,35,40,45,50, or 60 (Single dose)	SD	↓LH after 3h	Opioid Antag.	Cicero et al., 1993
Morphine	pellet sc (50-75ng/ml in blood), d25-46	SD	↓LH&sT, ↓testes&sv	Opioid agonist	Cicero et al., 1991
Naloxone	1 mg/kg-25 mg/kg,sc, d25, 30, or 35	SD	no△ LH after 3h	Opioid Antag.	Cicero et al., 1993
Naloxone	d35, sc, 5 mg/kg	SD	↑sLH, 30 min later	Opioid Antag.	Sylvester et al., 1985
Naloxone	d40 and 55, sc 2.5-5.0 mg/kg	Wistar	↑sLH,↓sPrl 15 min. later	Opioid Antag.	Aubert et al., 1988
Naloxone	1 mg/kg,sc,d40-60	SD	↑sLH after 3 h	Opioid Antag.	Cicero et al., 1993

TABLE 3 (continued)
Effects of Prepubertal Administration of Pharmacological and Environmental Chemicals on Pubertal Endpoints

Treatment	Dose (duration and route)	Strain	Effect	Putative Mechanism	Ref.
Naloxone	1.0 mg/kg d45, 50, 55, 60 65, sc	SD	↑sLH	Opioid Antag.	Nazian, 1992
Naloxone	2.5mg/kg, d51, or d52...58, single ip or iv	Wistar	↓sT and DHT 1 h later	hypoPRL ∴ ↓LH receptors; opioid antagonist	Stojilkovic et al., 1987
Naloxone	2.5 mg/kg, ip d37-44 (single)	Wistar	↑sLH	Opioid Antag.	Stojilkovic et al., 1987
	d51 to d58 (single)		↑sLH, ↓sPRL, ↓T+DHT	effect on PRL and subseq. T	Maric et al., 1987
Naltrexone	d55, 20mg/kg,ip	SD	↑sT (2 h later)	Opioid Antag.	Emanuel et al., 1998
Cimetidine	d20 and 28		↑sPRL	H2 histamine antagonist	Becu and Libertun, 1983
Ethanol	d35, 3 g/kg, i.p.	SD	↓GH at 1.5, 3,6, and 24 h post-inj	Disrupts eop control of GnRH	Tentler et al., 1997
Ethanol	d35, 2g/kg, ip	SD	↓sLH 2h later	Disrupts eop control of GnRH	Emanuele et al., 1998
Ethanol	d41, 3 g/kg, i.p.	SD	↓GH at 1.5, 3,6, and 24 h post-inj	Disrupts eop control of GnRH	Tentler et al., 1997
Ethanol	d45, 2g/kg,ip	SD	↓sT, ↓sLH (2h later)	Disrupts eop control of GnRH	Emanuele et al., 1998

Ethanol	d51, 3 g/kg, i.p.	SD	↓sT at 6 h post-inj, ↓GH at 1.5, 3,6, and 24 h post-inj	↓IGF-1 has been rep., leads to ↓GnRH, ↓LH leads to ↓sT	Tentler et al., 1997
Ethanol	d55, 2g/kg,ip	SD	↓sT, ↓sLH, GnRH mRNA (2h later)	Disrupts eop control of GnRH	Emanuel et al., 1998
Ethanol	d25-63, 29g/kg/day, in liq. diet	SD	↓sT, ↑sLH, ↓SVwt, ↓Testis wt, ↓hyp I-βE sac d63	Disrupts eop control of GnRH	Cicero et al., 1990
Estrogen Modulators					
Methoxychlor	**25 and 50 mg/kg/d** (d21-**80** g)	**LE**	↑pit PRL sac d80	weak estrogen and antiandrogen	Gray et al.,1988
Methoxychlor	100 mg/kg/d (d21-**80** g)	LE	↓pit wt. and sTSH ↑pFSH, pPRL, pTSH delayed PPS, also ↓BW. sac d80	weak estrogen and antiandrogen	Gray et al., 1988
Methoxycholor	400 mg/kg/d (d21-**80** g)	LE	delays PPS one month sac d80	weak estrogen and antiandrogen	Gray et al., 1989
4-OHE2	0.1 ug/h for 6d d26-31 Os. Minipump	Wistar	↓sFSH and testis wt. sac d31 (1700h)	estrogen metabolite	Ball et al., 1981
Estradiol	0.1 ug/h for 6d d26-31, Os. Minipump	Wistar	↓sFSH and testis wt sac d31 (1700)	estrogen metabolite	Ball et al., 1981

TABLE 3 (continued)
Effects of Prepubertal Administration of Pharmacological and Environmental Chemicals on Pubertal Endpoints

Treatment	Dose (duration and route)	Strain	Effect	Putative Mechanism	Ref.
2-OHE2	d35, 50 and 100ug sc	SD	Increase sLH (6h later)	estrogen metabolite	Naftolin al.,1975
<u>Androgen Modulators</u>					
Testosterone Proprionate	5&10 ug/100g BW d5-30, sc	Wistar	↓Testes wt (10ug) delay PPS &sperm. in Ppsmear	androgen	Freitag and Docke, 1987
Diethylhexylphthalate (DEHP)	2g/kg/day,g (d42-56)	Wistar	↓T, Testes, vp,sv wt ↑Liver wt.	antiandrogenic	Oishi, 1985
Dibutyl Phthalate (DBP)	500 and 1000 mg/kg/d (d21-57,g)	LE	↑serum LH and FSH	plasticizer antiandrogenic	Gray et al., 1988
Di-n-pentyl phthalate (DPP)	7.2 mmol/kg/d,g (d35-39)	SD	↑Testicular P-450 enzymes, ↓17βHSD	antiandrogenic	Foster, 1983
Vinclozolin	10,30 and 100mg/kg/d (d22-54,g)	LE	↑serum LH	antiandrogenic	Monosson et al., 1999
Vinclozolin	100 mg/kg/d (d22- 54 ,g)	LE	↑T and 5αA3αdiol delays PPS 4-7 days	antiandrogenic	Monosson et al., 1999
Vinclozolin	100mg/kg d21-23, g	Aderly Park	d24↑Liver wt	antiandrogen	Ashby and Lefevre, 1997
"	d21-27	"	d28↓Epi,sv wt	"	"
"	d21-34	"	↑Testes wt. ↓Prostate	"	"

Anastrozole	25 mg/kg d21-23	"	d24 ↑Liver ↓sv, prost.	aromatase inhib	"
	d21-27	"	d28 ↑Liver		"
	d21-34	"	d35 ↑Liver&kid ↓sv, prost.		"
Cyproterone acetate	25 mg/kg d21-27,g	"	d28, ↓testes, epi, sv, prost.	5α-reductase inhib.	Ashby and Lefevre, 1997
"	d21-34	"	d35 ↓testes, epi,sv	"	"
p,p'-DDE	100 mg/kg/d (d21-57, g)	LE	delays PPS(5d) ↑BW, no△T sac d57	antiandrogenic	Kelce et al., 1995
Linuron	40 mg/kg/d g	LE	delays PPS 2.5 days	antiandrogenic(weak)	Gray et al., 1999
Thyroid Hormone Modulators					
Barbital	0.1% in water d28-56	SD	↑sTSH, ↓pTSH, ↓T4 ↑Thyroid Wt. sac d 56	induction of metab. enzymes	Ching, 1981
Propylthiouracil (PTU)	0.05% in water d28-56	SD	↑sTSH, ↓T4, ↓BW, ↑Thyroid Wt.	inhib. T4 to T3	Ching, 1981
methimazole	d40-d140 In drinking H_20	Wistar	140 d sac ↓sLH,T4,T3,PRL And sT	induction of metab enzymes	Valle et al., 1985

TABLE 3 (continued)
Effects of Prepubertal Administration of Pharmacological and Environmental Chemicals on Pubertal Endpoints

Treatment	Dose (duration and route)	Strain	Effect	Putative Mechanism	Ref.
Prolactin Modulators					
ectopic pituitary	d21-PPS	Wistar	↑sPRL, adv. PPS sac on PPS	increase PRL	Aguilar et al., 1988
ectopic pituitary	d30-51	Holtzman	↓sv,vp,dp sac d51	"	Negro-Vilar et al., 1977
ectopic pituitary	d21-28,35,42,49,56	Wistar	adv. PPS(1 wk) ↑vp&sv	"	de Jong and van der Schoot, 1979
Ovine Prolactin	5,10,25,50, or 500 ug/day, d30-40,sc	Wistar	↑sLH, 40d sac	"	Baraño et al., 1982
Ovine Prolactin	500 ug/day "	Wistar	↑Testes, prostate, epid. wts., ↓and. receptors, d40 sac	"	Baraño et al., 1982
Ovine Prolactin	25,50 and 500 "	Wistar	↑3 αdiol, d40 sac	"	Baraño et al., 1982
bromocriptine	1mg/kg, d23-d32	SD	↓PRL,↓pPRL ↓Lhr,↑pLH, ↑GnRHr	DA agonist	Huhtaniemi et al., 1984
bromocriptine	d30-d40, 0.75, 1,5, Or 3.0 mg/kg/day,sc	Wistar	↓sPRL,no△sLH, ↓sT,DHT and 3 αdiol,	By ↓Prl Brom indirectly infl. steroidogenesis	Suescun et al., 1985

			In vitro: ↓resp.to submax HCG saced at 40d	by ↓LH receptors on testes	
Bromocriptine	3 mg/kg, sc d9-24	Wistar	hypoPRL, ↑sT/ DHT, ↑dev. of Leydig cells	↓Prl favors prolif.	Kovacevic et al. 1982
Bromocriptine	d9-45	"	↑dev. Leydig cells	(Prl ↓ 5-αR)	"
Bromocriptine	3 mg/kg,sc d9-72	Wistar	↓sT/DHT ↓dev.Leydig cells ↓vp,dp, sv wt.	post-puberty ↑sPRL is needed for maint. of high androgens	Kovacevic et al. 1982
Pimozide	20 mg/kg, sc d22-32	Wistar	on d29, ↑Prl d120, ↑LP wt Prostatitis (LP)	DA antagonist (hyper-Prl)	Stoker et al., 1999
Bisphenol A	50 mg/kg, sc d22-32	Wistar	on d29, ↑Prl d120, ↑LP wt Prostatitis (LP)	Weak estrogen (hyper-Prl)	Stoker et al., 1999
17β-Estradiol	implant (80-100pg/ml in serum on d29, d22-32	Wistar	on d29, ↑Prl d120 LP Prostatitis	Estrogen (hyper-Prl)	Stoker et al., 1999
chlorpromazine	d20, 40 or 60 25 mg/kg,ip	SD	↑sPRL, max 15min in 20d, 30 min in 40&60	short acting phenothiazine tranquilizer	Bohnet et al., 1976
fluphenazine enanthate	d20,30 or 60, 25 mg/kg, sc	SD	d20 less resp. d30&60 stayed Elev. 10days	Long-acting phenothiazine	Bohnet et al., 1976

TABLE 3 (continued)
Effects of Prepubertal Administration of Pharmacological and Environmental Chemicals on Pubertal Endpoints

Treatment	Dose (duration and route)	Strain	Effect	Putative Mechanism	Ref.
Pineal Function Modulators					
Melatonin (MT)	100ug, sc, d20-40 In afternoon only (Late photophase)	Wistar	↓GnRH receptors ↓pFSH and sFSH ↓sv and testes wt. ↓sLH and T	indolamine	Lang et al., 1984
Growth Hormone and Growth Factor Modulators					
antiserum to GRF	d15 to 39,sc	SD	d40 and 50: ↓testes,sv, IGF-I,sFSH,pLH,pFSH delayed spermatogenesis	low IGF-I	Arsenijevic et al., 1989
Other					
Lead Acetate	56.9 ug/dl in blood d22-?? , in water	SD	↓sLH, ↑LHβ mRNA d74 sac	Pit effect	Ronis et al., 1996
Lead acetate	d42-70 in water	Wistar	d70no change in sT or sperm	Pit effect	Sokol and Berman, 1991
	d52-82 in water		↓sT, ↓sperm	"	Sokol and Berman, 1991
Lithium Chloride	d35to 50, 55, or 60 2.0 mg/kg/day, sc	Wistar	50,55, 60d: ↓sLH,sFSH, sPRL,and sT 55 and 60d: ↓testes, prostate, SV ↓3β-HSD, 17β-HSD	Pit effect/ inhib. steroido-genesis	Ghosh et al., 1991

Other

Δ^8 THC	4mg/kg,i.p., d16-40(eod)	Ivanovas	↓T,↓DHT,↓sFSH ↓sLH, sac d40	Inhibit gonadotropin secretion	Gupta and Elbracht, 1983
Δ^9 THC (less recovery)	4mg/kg,i.p., d16-40(eod)	Ivanovas	↓T,↓DHT,↓sFSH ↓sLH, sac d40	"	Gupta and Elbracht, 1983
Δ^9 THC	1 mg/kg, i.p, 3 X per wk d21-51	SD	↓sLH, ↓sGH sac d51	"	Collu et al., 1975
Δ^9 THC	10 mg/kg,i.p. 3 X per wk d21-51	SD	↓sLH, ↓sGH ↓prostate wt. sac d51	"	Collu et al., 1975
Δ^8 THC	4mg/kg,i.p., d16-87(eod)	Ivanovas	↓BW,↓T,↓DHT ↓sFSH,↓sLH sac d87	Decreasing LH and FSH release at pit level	Gupta and Elbracht, 1983
Δ^9 THC (greater effects)	4mg/kg,i.p., d16-87(eod)	Ivanovas	↓BW,↓T,↓DHT ↓sFSH,↓sLH sac d87	Decreasing LH and FSH release at pit level	Gupta and Elbracht, 1983
Carbendazim	200 and 400 mg/kg/d (d21- , g)	LE	↑pLH, pFSH,sFSH	fungicide Testicular toxicant	Gray et al., 1988

Note: g = gavage; sc = subcutaneous; ip = intraperitoneal; eod = every other day; max = maximum; SD = Sprague Dawley; LE = Long Evans Hooded; s = serum; p = pituitary; r = receptor; d = PND; h = hour; sac = sacrifice; TH = tyrosine hydroxylase. For other abbreviations, see Abbreviations.

of corticosteroids (mineralo- and glucococorticoids) and medullary (epinephrine) hormones, an increase in pituitary ACTH secretion or the accompanying decrease in body growth. In female rats, reports suggest that ACTH may exert inhibitory effects on the reproductive system via a direct action on the hypothalamic/pituitary axis (Macfarland and Mann, 1977). In males, exogenous ACTH inhibits gametogenic development in the immature rat, which leads to cellular degeneration of the testes (Kapil et al., 1979).

C. The Pineal Gland

Two types of pineal secretory products have been described: indoleamines and polypeptides. The pineal gland plays a role in transducing environmental information such as light or season into secretory signals (Wurtman and Axelrod, 1966). The pineal influence is mostly inhibitory and the signal is primarily carried out by the secretion of the indoleamine melatonin. Melatonin is the principal indoleamine responsible for the timing of sexual maturation in the rat (Lang et al., 1984).

Inasmuch as the rat does not have a breeding season, it would appear that at constant laboratory conditions (such as temperature, long photoperiods and ad libitum feeding), the pineal gland has little function (Reiter, 1980). However, the removal of the pineal gland in the male rat can advance sexual maturity, and a daily injection of melatonin can delay puberty, reduce the weight of the testes and seminal vesicles, decrease serum testosterone, LH and FSH and decrease the number of GnRH receptors on the pituitary, but only if administered during the late photophase (9 to 12 h after lights on) in the juvenile-peripubertal period (Aubert et al., 1988; Lang et al., 1985). The exact mechanism of action of melatonin administered prepubertally is not known. However, a few studies have implicated a direct pituitary gland or gonadal site of action (Ellis, 1972; Martin and Sattler, 1982). It appears that testosterone is involved in the regulation of melatonin receptor density in the suprachiasmatic nuclei and pars tuberalis in the male rat (Zitouni et al., 1996).

There has been much concern about effects of melatonin during prepuberty in humans, as it has been linked to pubertal development in animal models. Because of these concerns, melatonin is not recommended for use as a sleep aid in individuals under age 18, or in lactating or pregnant women (Arendt, 1997). Elevated melatonin levels are thought to function during prepuberty to keep the HPG quiescent, exerting an inhibitory effect on pubertal development (Cavallo and Dolan, 1996; Commentz et al., 1997). The levels then fall during puberty and adulthood.

Although few studies have examined the effects of toxicants on pineal gland functioning in the rat, several investigators have evaluated effects following melatonin administration during the prepubertal period. For example, melatonin analogs, such as *N*-acetyl-serotonin and 5-hydroxytryptophol, administered during the prepubertal period have no effect on the HPG axis, as does the administration of melatonin from PND20-40 (Table 3; Lang et al., 1983). As discussed, melatonin administered from PND 20-40 (in the late photophase only) resulted in a decrease in serum gonadotropins, likely due to a decrease in GnRH receptors (Lang et al., 1984). These effects during puberty in the male may be mediated by an increase in NPY, because it has been shown that NPY was increased in male rats with delayed sexual maturation induced by injections of melatonin (Corder et al., 1992).

V. THYROID

The thyroid gland is a two-lobed endocrine organ that secretes thyroid hormones that are critical for normal growth and differentiation and regulate metabolism in most organ systems. The precursors of thyroid hormone biosynthesis are iodide and thyroglobulin. Biosynthesis and secretion of the thyroid hormones are under a feedback control of the hypothalamic-(TRH-thyrotropin-releasing hormone)-pituitary (TSH-thyroid stimulating hormone)-thyroid axis (thyroid hormones-T3 and T4). TSH regulates the synthesis of the two thyroid hormones, thyroxine (3,5,3′-tetraiodothyronine or T4) and 3,5,3′-triiodothyronine (T3), and stimulates their release into the circulatory system where they bind to transporting proteins (in the rat, either transthyretin or albumin) for peripheral distribution. T4 is converted to the active form, T3 , by

Type I 5′-deiodinase in the liver or Type II 5′-deiodenase in the brain, pituitary, and brown adipose tissue. The T3 then binds to its intracellular nuclear receptor and stimulates gene transcription.

Typically in male rats, serum TSH increases progressively from days 30 to 50 and is lower by day 60, while pituitary TSH increases from days 21 to 50 and remains constant through day 80 (Table 2) (Simpkins et al., 1976). Administration of exogenous TRH induced a significant elevation in serum TSH at 40 days, but was less effective on day 25 (Simpkins et al., 1976). This suggests that serum TSH levels increase in male rats at about the time of puberty and then decline, and that changes in the response to TRH may account in part for the increase in serum TSH levels during development.

Thyroid hormones, besides being essential for normal growth and development, have been reported to be protective of gonadal functions in the male rat. For example, Valle et al., 1985, have shown that an induced hypothyroidism from prepuberty to adulthood (by treatment with methimazole from PND 40 to 140) caused delayed sexual maturation, with atrophy of the testis and accessory sex glands, reduced level of gonadotrophins, prolactin, and testosterone and severe inhibition of gametogenesis. This delay in sexual maturation appeared to be caused by thyroid alterations of LH-mediated testosterone secretion. In addition, thyroidectomy of immature rats severely inhibits gametogenesis and interstitial cell development (Chowdury et al., 1984). For a discussion of toxicant-induced hypothyroidism see the Toxicant Effects on Thyroid Function section.

Specific receptors for T3 have been localized in Sertoli cells, with a decrease in number of receptors from PND 7 to 35 and no change in binding affinity (Palmero et al., 1993), suggesting a possible role of T3 in the onset and patterning of Sertoli cell functioning. A hypothyroid condition results in a delay in this postnatal decrease in testicular T3 receptors. Treatment of newborn rats with T3 increased Sertolli cell proliferation and hastened Sertoli cell differentiation, resulting in an increased number of Sertoli cells and germ cells (Ulisse et al., 1998), as well as an increased seminiferous cord and testicular size (Jannini et al., 1993). T3 may also regulate the basement membrane surrounding seminiferous epithelium (Ulisse et al., 1998).

VI. THE TESTIS

A. Perinatal/Postnatal Development

Sexual differentiation in the male is determined during fetal life, with the seminiferous cords being formed by gestational day 13 (GD 13) (Waites et al., 1985). Leydig cells are found in the fetal testes and are responsible for androgen synthesis. In pubertal and adult animals, testosterone synthesis is regulated/stimulated by the binding of luteinizing hormone (LH) to the luteinizing hormone receptors (Lhr). However, even though luteinizing hormone receptors are present on the Leydig cell by GD 15.5 with maximal levels attained by birth (Warren et al., 1984), circulating LH levels are low prior to the testosterone surge that takes place at approximately GD 19 to 20. This indicates that another factor other than LH is likely responsible for the initial testosterone surge during gestation. After birth the LHr number in the testes increases in parallel with testicular testosterone content and the number of Leydig cells in the interstitial region of the testes. Sertoli cells are located within the seminiferous tubules of the testes and are the sine qua non of spermatogenesis. These cells undergo rapid division at the end of gestation and then cease to divide after the first 2 weeks postnatally. The Sertoli cells are stimulated by follicle-stimulating hormone (FSH) and possess FSH receptors (FSHr) as early as GD 17.5. It is evident that the endocrine system in the fetal male is already functional just prior to birth. The activation of hormone action during this time is essential for the development of the reproductive tract and consequent fertility in adulthood.

B. Leydig Cells/Androgen Synthesis

Leydig cells (LC) are located between the tubules in the interstitial portion of the testes and are the primary source of testosterone production in the male. During late gestation, fetal LCs begin producing testosterone at approximately GD 15, with peak production occurring between GD 18 and GD 20. These fetal LCs atrophy between PND 15 and 19 and do not divide postnatally. Adult LCs are not derived from the fetal LC, but instead stem from undifferentiated progenitor cells. There is still some controversy as to the cell origin of these progenitor

cells, but increasing evidence indicates that spindle-shaped cells in the interstitial area of the testis are undifferentiated progenitor cells (de Kretser and Kerr, 1994) and arise from proliferation of mesenchymal-like stem cells at approximately 2 to 3 weeks after birth (Hardy et al., 1989). There appears to be three stages of postnatal LC development (Benton et al., 1995) with a maximal concentration of progenitor LCs at PND21, followed by the immature LCs at PND35 and a full population of adult Leydig cells by PND 90. It is speculated that the differentiation of these cells is hormonally influenced by gonadotropins such as LH, which has the capacity to influence androgen secretion at PND 25, 33, 40, and 60 days of age with a maximal response at PND 40 and 60 (Moger, 1977). In a more recent study by Shan et al., 1997, LH, as well as androgens, were found to regulate androgen receptor levels in LCs with a peak sensitivity to these factors occurring at PND 35 (immature LCs). It is hypothesized that androgens and LH stimulate the conversion of progenitor LCs into immature LCs, whereas insulin-like growth factor-1 (IGF-1) is thought to regulate the differentiation of the immature LCs into adults LCs (Benton et al., 1995). This theory of IGF-1 regulation is strengthened by *in vitro* data that indicate that immature LCs have IGF-1 receptors and an elevated stimulatory response to IGF-1 (Hardy et al., 1991).

As there are differences in the regulators of the three phases of LC development, there are also differences in androgen synthesis between these cells. From PND 5-25 the number of LCs (progenitors) in the testis increases rapidly, but there is no significant change in testosterone concentration in the testis (Ketelslegers et al., 1978). This is due to the fact that these cells are predominantly producing the 5 α-reduced androgen, androsterone, instead of testosterone (Ge and Hardy et al., 1998). Even though androgens are produced by the progenitor LCs, their capacity for steroidogenesis is still much lower as compared to immature and adult LCs. From PND 28 to 40 the newly derived immature LCs undergo cell division (Hardy et al., 1991) and the primary androgen produced at this time is 3 α-androstanediol (Ge and Hardy, 1998). This is accompanied by an initial rise in plasma testosterone levels between PND 25 to 35, which is then followed by a much larger increase in testosterone levels between PND 35 to 55 (Ketelslegers et al., 1978) when the adult LCs predominate (Monosson et al., 1999; see also Figure 1A).

C. Steroidogenesis

1. Mediators

Postnatally, the activity of the hypothalamic/pituitary/gonadal axis becomes fully operational and involves a series of regulators that initiate and maintain steroidogenesis at puberty. However, the precise mechanism that stimulates the onset of puberty and steroidogenesis is still uncertain. One factor that is thought to be involved in androgen synthesis postnatally is LH, which is released from the pituitary and transported to the testis where it binds to the LHr. This binding results in an increase in cyclic AMP production (Marsh, 1976), which acts by phosphorylating a number of proteins leading to an increase in androgen synthesis. In one study, LH and DHT separately were not able to stimulate testosterone secretion; however, LH and DHT together had a significant effect (Hardy et al., 1990). As mentioned previously, serum LH levels during puberty remain controversial due to lack of corroborating data from birth to puberty, with some studies reporting an increase in LH during puberty (Dohler and Wuttke, 1974; Ketelslegers et al., 1978; Lee et al., 1975) and others reporting very little change in circulating LH (Chiappa and Fink, 1977; Kamberi et al., 1980., 1980; Ketelslegers et al., 1978; Matsumoto et al., 1986; Ojeda and Urbanski, 1994). Apart from the controversy over circulating LH levels, it is clear that LH is involved in LC differentiation during puberty (see Leydig Cell section).

Another hormone thought to play an important role in androgen synthesis and onset of puberty is FSH. Treatment with FSH appears to increase LC numbers and because seminiferous epithelium is the target of FSH, this suggests that secretory factors from the seminiferous tubules may mediate LC maturation (de Kretser and Kerr, 1994). As mentioned, it is also speculated that FSH induces the appearance of LHr, which may be asso-

ciated with increased Leydig cell numbers instead of increased number of LHr per cell. An increase in LHr number is followed by an increase in steroidogenic responsiveness of the LC during LH or hCG stimulation. The presence of FSH thus appears to indirectly regulate the process of androgen synthesis in the testes.

Additionally, it has been shown that three Sertoli cell proteins stimulate LC steroidogenesis: inhibin, TGF-α and insulin-like growth factor-I (IGF-I) (Skinner et al., 1991). However, all three proteins are also secreted by the LC and elsewhere, so it is unclear whether they act in an autocrine or paracrine fashion (Hardy et al., 1991). Support for the actions of IGF-I in LC maturation is strong due to the presence of receptors for IGF-I on Leydig cell progenitors. Testicular levels of IGF-I are also highest during puberty when it stimulates proliferation of mesenchymal cells, immature LCs, and adult LCs (Lin et al., 1987; Lin et al., 1988; Nagpal et al., 1991).

2. Enzymes Involved in Steroidogenesis

There are several enzymatic reactions and cofactors involved in the synthesis of testosterone. The immediate source for the steroid ring of testosterone is a depot of cholesterol in the cytoplasm that is derived from low-density lipoprotein (Hall, 1994). The majority of the enzymes involved in the conversion of cholesterol to testosterone are members of a family of monooxygenase enzymes known as cytochrome P-450s. These enzymes are important in the biotransformation/elimination of xenobiotics and in the inactivation of endogenous substrates. In addition, several P-450s play an integral part in the synthesis pathway of steroid hormones involving four specific enzyme steps: (1) hydroxylation, (2) dehydrogenation, (3) isomerization, and (4) C-C cleavage (Hall, 1994).

Other enzymes involved in steroid synthesis include the 3β-hydroxysteroid dehydrogenase complex (3β-HSD: 3β-hydroxy steroid dehydrogenase/delta 5-delta 4 isomerase), which is essential in the biosynthesis of all biologically active steroids. Using immunocytochemistry, LCs showed increasing 3β-HSD activity from GD 17 through GD 21, and the cells were arranged in clusters between seminiferous tubules (Dupont et al., 1993). The number and size of cells expressing 3β-HSD activity decreased greatly at PND 1 and did not start to rise until between PND 12 and 19. There is an even greater increase in 3β-HSD activity prior to puberty, between PND 19 and 32 and another large increase in 3β-HSD activity during the onset of puberty. These increases in enzymatic activity are mirrored by increases in androgen secretion, indicating a direct relationship between 3β-HSD activity and androgen production (Dupont et al., 1993).

Another important enzyme in the steroidogenic pathway is 17β-hydroxysteroid dehydrogenase (17β-HSD). The activity of 17β-HSD is increased slightly later than 3β-HSD beginning at approximately PND-22 with a rapid increase between PND 37 and 58. This increased activity also closely follows testosterone synthesis (Payne et al., 1977) and may be correlated with changes in FSH secretion. As mentioned earlier, it is thought that FSH secretion is a major factor in testicular maturation and that the testes appear to be conditioned by FSH with an increased responsiveness to LH, resulting in greater testosterone production. Thus, it is speculated that FSH may be involved in the regulation of 17β-HSD (Payne et al., 1977).

3. Steroidogenesis in Brain

Several studies in the rat have reported key steroidogenic enzymes in peripheral/nongonadal tissues. Some of these enzymes include 20, 22-demolase: cholesterol side chain cleavage cytochrome P450 (P450scc) that converts cholesterol to pregnenolone in the rat brain and 17 α-hydroxy/C-17,20-desmolase cytochrome P450 (P450c17) that converts pregnenolone to progesterone with subsequent bond scission to dehydroepiandrosterone and androstenedione, respectively. These enzyme activities are also expressed in the rat liver and gonads in both sexes (Vianello et al., 1997). During the second postnatal week there is a peak in expression in the liver that is higher than in the testis and gradually declines below testis activity by the fourth postnatal week. Conversely, activity in the testis is relatively high prenatally (GD 18), decreasing at birth and starting a steady increase again at PND 16 through puberty and adulthood

(Vianello et al., 1997). Additional studies have revealed that there is also P450c17 activity in the duodenum that is comparable to that in the testis (Dalla Valle et al., 1996). These studies are preliminary, and their role in androgen activity in puberty is not clear.

Other steroidogenic enzymes in the male prepubertal brain are also major contributors to the sexual development of the male rat and include 5α-reductase, responsible for the conversion of testosterone to DHT, and aromatase, responsible for the conversion of testosterone to 17β-estradiol. During gestational development in the male, aromatase activity plays an integral role in structural organization of the hypothalamus, which can effect sexual behavior later in development. It is most important during early development, and the completion of reproductive maturity is associated with a decrease in hypothalamic aromatase and 5α-reductase at approximately PND 90 (Lephart and Ojeda, 1990). The activity of brain aromatase levels is thought to be regulated by androgens, but this regulation appears to be lost during early adulthood. Similarly, the activities of 5α-reductase increase in the brain between PND 20 to 40 but decrease between PND 40 to 60 (Goldman and Klingele, 1974). This decreased activity may play a part in the decreased sensitivity to steroid negative feedback that occurs at the completion of puberty (Lephart and Ojeda, 1990).

D. Androgen Activity

1. Steroid Hormone Receptors

Positive immunohistochemical staining of the androgen receptor (AR) was found as early as GD 15 in the mesenchymal and epithelial cells of the mesonephros, with weak staining observed in the interstitial cells (You and Sar, 1998). However, in this same study the Sertoli cells did not express the receptor until PND 5. This expression of the AR at various times and tissues may explain the differential effect of certain chemicals at various developmental stages. An AR has also been described in PND 20 rat testes that correlates with a rise in DHT concentration at this time. Conversely, the maintenance of the AR in the ventral prostate (Sullivan and Strott, 1973) does not appear to be androgen-dependent. The concentration of nuclear androgen receptors (ligand bound) in the testes decreased from PND 15 to PND 25 and then increased steadily until full sexual maturity between PND 60 and PND 90 (Buzek and Sanborn, 1988). In another study by Shan et al. (1992), the highest concentration of AR mRNA was identified in immature LC at PND 35, the next highest found in the progenitor LC at PND 21 and the lowest level in the adult LC at PND 90. In contrast, AR mRNA levels are highest at PND 90 in the Sertoli cells and are regulated by LH, FSH and androgens, whereas these factors are not effective in the regulation of the AR in LCs at this age (Shan et al., 1997). These factors play a more important regulatory role in AR levels in the LC at PND 21 and 35 (Shan et al., 1997). These findings indicate that there are several distinct mechanisms responsible for the regulation of AR expression during development.

E. Androgen Function

Androgens are critical in the development of the male reproductive tract, in the feedback inhibition of hypothalamus-pituitary, for the maintenance of sex accessory organs, and in the stimulation and maintenance of spermatogenesis during puberty. The presence of testosterone is necessary for the development of the testes, epididymis, vas deferens, seminal vesicles, levator ani/bulbocavernosus (LABC), and other muscles and is also responsible for stimulating the inguinoscrotal descent of the testes (PND 15). In comparison, DHT initiates postnatal regression of nipples/areolas and is a key hormone in the development and maintenance of the prostate, urethra, external genitalia, and preputial separation (also see section on prolactin for prostate) (Luke and Coffey, 1994; Mylchreest and Foster, 1998). The AR allows these androgens to mediate male sexual development. Testosterone and DHT are the two androgens that bind with the highest affinity to the AR and are the most biologically active. Although their affinity to the receptor is similar, the kinetics of dissociation of testosterone and DHT are very different, with the dissociation of testosterone about three times faster than DHT. These differences may stem from the fact that most of the testosterone-sensitive tissues in the reproductive tract are derived from the Wolffian duct and are close in proximity to the testes

where there is a high concentration of testosterone and very low 5α-reductase activity in the Wolffian duct before puberty (George and Wilson, 1994).

VII. TOXICANT EFFECTS ON MALE PUBERTAL DEVELOPMENT AND THYROID FUNCTION

The following section includes a review of the literature for those chemicals, including both pharmacological and environmental compounds, known to alter male reproductive development and thyroid function. Although perinatal and neonatal exposures can also have effects on pubertal development in the rodent, this discussion will be limited primarily to those studies in the rat that report similar exposure parameters as the male pubertal protocol, with exposure during the juvenile and peripubertal period (PND 23-53). The reader is encouraged to refer to Table 3 as a complete reference for the toxicant-induced alterations discussed in this section and Table 4 for a general summary of the effects reported for particular receptor modulators.

A. Testis/Reproductive Tract Alterations

Development of the size of the penis and cornification of the epithelium of the prepuse and preputial separation in immature rats are regulated by androgens (Marshall, 1966). Administration of testosterone causes an increase in penile size and accelerates preputial separation in immature rats (Lyons et al., 1942). In fact, general growth of the organ can be induced by local application of testosterone (Wigodsky and Green, 1940). The separation of glans and prepuce is also prevented by castration in both rats and mice (Lyons et al., 1942). In this regard, balano-preputial separation (PPS) has been used as a marker of puberty in the male rat. PPS is a cornifying process that leads to cleavage of the epithelium forming the stratified squamous lining of the prepuce of the penis. This cleavage is a sign of puberty and is an essential prerequisite for acquisition of complete ejaculation (Wisner et al., 1983). This process also represents a reliable noninvasive indicator of chemical-induced perturbation to rat pubertal development (Gaytan et al., 1988).

B. Estrogen-Induced Alterations

Estrogens act by delaying PPS in the juvenile male rat, an effect that can persist for greater than 50 days of age (Monosson et al., 1999). However, delays of 3 to 5 days are more typical, with separation normally occurring at about PND 43. Subchronic methoxychlor administration prior to puberty delays the onset of puberty (PPS) in male rats at doses of 50 to 200 mg/kg/d in a dose-related manner (Anderson et al., 1995) and at doses of 50 and 150 mg/kg/d (Chapin et al., 1997). In addition, methoxychlor-treated males had smaller seminal vesicles and ventral prostates, lower epididymal and ejaculated sperm numbers, and reduced numbers of copulatory plugs (Anderson et al., 1995; Gray et al., 1989). These endpoints are affected not only by estrogens but by environmental antiandrogens, drugs that affect the hypothalamic-pituitary axis (Hostetter and Piacsek, 1977; Ramaley and Phares, 1983), prenatal exposure to TCDD, an AhR agonist (Bjerke and Peterson, 1994; Gray et al., 1995) and dioxin-like PCBs (Gray and Kelce, 1996). Thus, it is too premature to state that methoxychlor is acting only as an estrogen but perhaps also as an antiandrogen.

In contrast to the delay of PPS by estogenic compounds in the male rat, peripubertal estrogen administration accelerates the onset of the pubertal process in the female (vaginal opening or VO). In a 90-day study, Biegel et al., 1998, found that dietary adminstration of 17β-estradiol accelerated vaginal opening at 0.05 and 2.5 ppm (by 1.6 and 8.8 days, respectively), while 2.5 ppm estradiol affected reproductive markers, including a delay in preputial separation by 8.2 days. The endpoints of PPS and VO were more sensitive than were effects of estradiol β on fertility (down at 10 ppm) or ovarian (reduced corpora lutea at 2.5 ppm and cystic follicles at 10 ppm) or testicular (atrophy) histopathology. Females treated with estradiol at 2.5 ppm also displayed abnormal estrous cycles, resulting in a more constant estrus condition. Hence, these studies demonstrate that pubertal landmarks are sensitive to estrogenic disruption in both male and female rats, with the female more sensitive than the male.

Environmental estrogens or antiestrogens come from a variety of sources, including pesticides, plant products, and food storage containers. Some of these products bind to the estrogen receptor and mimic

TABLE 4
Summary of Effects on EDSTAC Endpoints in Pubertal Development and Thyroid Function Protocol

Chemical	Growth	Preputial Separation		Weights						Hormones[a]	Histology[b]
		Age	Body Weight	Testis	VP	SV	Epi.	Liv.	Thyr.		
GnRH											
Agonist		↓		↓	↓	↓				↓T	
Antagonist		↓		↓	↓	↓				↓LH, FSH,T	
Dopamine											
Agonist										↓PRL,LH	
Antagonist (or oPRL)		↓		↑	↑	↑	↑			↑PRL,LH	
Opioids											
Agonist				↓		↓				↓LH,T	
Antagonist										↑LH, ↓PRL,T	
EtOH				↓		↓				↓T,LH	
Androgen											
Agonist (low conc) (aromatizable)		↓		↓	↓	↓	↓			↓T,LH	
Agonist (high conc) (aromatizable)		↓		↑	↑	↑	↑				
Antagonist	↑	↑	↑	↓	↓	↓	↓	↑		↑T, LH	

Estrogen															
Agonist	↓		↑		↓	↓						↓FSH			
Antagonist															
Arom inhib. (or 5αR inhib)						↓		↓	↓	↓	↑				
Thyroid Hormone															
Inhib. of T4 to T3 or increased elim.	↓										↑	↑TSH; ↓T3,T4		↓TFCA;↑TFCH	

Based on studies with similar testing period.

[a]Hormones: LH-luteinizing hormone; FSH-follicle stimulating hormone; PRL-prolactin; T3,T4-thyroid hormones; TSH-thyroid stimulating hormone; T-testosterone

[b]Histology: TFCA- thyroid follicular colloidal area; TFCH- thyroid follicular cell height.

estrogen action. The estrogenic activity of Kepone (a pesticide also called chlordecone), octylphenol (a surfactant in detergents and pesticide formulations), some co-planar PCB's, and bisphenol-A (a monomer of polycarbonate plastics and a constituent of resins used in food packaging and dentistry) have been found to act as an estrogen agonists (Anderson et al., 1995; Olea et al., 1996; Schafer et al., 1999; Sonnenschein and Soto). One example of the effects of this binding in males was shown after occupational human exposure to Kepone which resulted in decreased sperm counts (review in Guzelian, 1992). Adverse testicular effects from Kepone were also identified in the adult male rat, demonstrated by the atrophy of the testes, which leads to reduced sperm counts (Reuber, 1979). The effects of many xenoestrogens on PPS in the rat are unknown, while other studies have been negative. Several investigators have reported on the effects of xenoestrogens such as nonylphenol (Chapin et al., 1998), bisphenol A (Cagen et al., 1999), and octylphenol (Gray and Ostby, 1999; Tyl et al., 1999) on pubertal landmarks in first-generation male and female rats. While nonylphenol and octylphenol, through gavage but not dietary administration, accelerated vaginal opening in females, none of the aforementioned chemicals delayed PPS or affected any other indices of reproductive function in the male. In contrast to these negative results, several different laboratories have reported that administration of methoxychlor produced similar delays in PPS in the male rat (Anderson et al., 1995; Chapin et al., 1997; Gray et al., 1989) (Figure 2b). However, methoxychlor metabolites are AR antagonists, as well as ER agonists, so the delay in PPS may be related to the antiandrogenicity rather than the estrogenicity of this compound (also see Leptin/Growth section, last paragraph).

C. Antiandrogen-Induced Alterations

The effects of estrogenic substances on pubertal development can be distinguished from antiandrogens by dosing animals with toxicants prior to and during puberty and determining their effect on the age at puberty in both male and female weanling rats. Delays in male puberty result from exposure to both estrogenic and antiandrogenic chemicals, including methoxychlor (Gray and Ostby, 1998), vinclozolin (Anderson et al., 1995; Monosson et al., 1999), and *p,p′* 1,1-dichloro-2,2*bis* (*p*-chlorophenyl)ethylene) (*p,p′*-DDE) (Kelce et al., 1995). Exposing weanling male rats to the antiandrogenic (AR antagonists) pesticides *p,p′* DDE or vinclozolin delays pubertal development in weanling male rats as indicated by delayed PPS and increased body weight (because they are older and larger) at puberty.

In contrast to exposure to estrogenic substances, antiandrogens do not inhibit food consumption or retard growth (Anderson et al., 1995). Antiandrogens cause a delay in PPS and affect a number of endocrine and morphological parameters, including reduced seminal vesicle, ventral prostate, and epididymal weights. In studies of vinclozolin, increases in serum LH were a sensitive response to this antiandrogen, whereas serum LH is not increased in males exposed to *p,p′* DDE during puberty (Kelce et al., 1995). It is clear that some xenobiotics and drugs with antiandrogenic activity elevate serum LH and testosterone levels and subsequently increase testes weight (Monosson et al., 1999), while other antiandrogens that induce a similar delay in PPS fail to alter LH or testosterone levels (Kelce et al., 1995). Chronic elevation of LH causes hyperplasia of the Leydig cells as in Cook et al., 1999, and increase intersitial fluid volume, which contribute to the observed increase in testes weight.

Preputial separation and sex accessory gland weights are sensitive endpoints. However, a delay in PPS is not pathognomonic for antiandrogens. Antiandrogenicity of these compounds is mediated through antagonistic binding to the androgen receptor; however, as discussed, pubertal alterations may also be caused by chemicals that disrupt hypothalamic-pituitary function (Huhtaniemi et al., 1986), or steroid hormone synthesis or metabolism. Compounds such as dibutyl phthalate (DBP), dipentylphthalate (DPP), and diethylhexyl phthalate (DEHP) produce antiandrogenic effects characterized by testicular atrophy, decreased serum and testicular testosterone levels, and alterations in steroidogenic enzymes (Foster et al., 1983; Gray et al., 1982; Mylchreest and Foster, 1998; Oishi, 1985; Srivastava and Srivastava, 1991). Male rats (PND 35) exposed by gavage to 7.2 mmol/kg/d for up to 4 days had significantly smaller testes weights, decreased activity of two testicular steroidogenic enzymes, 17α-hydroxy-

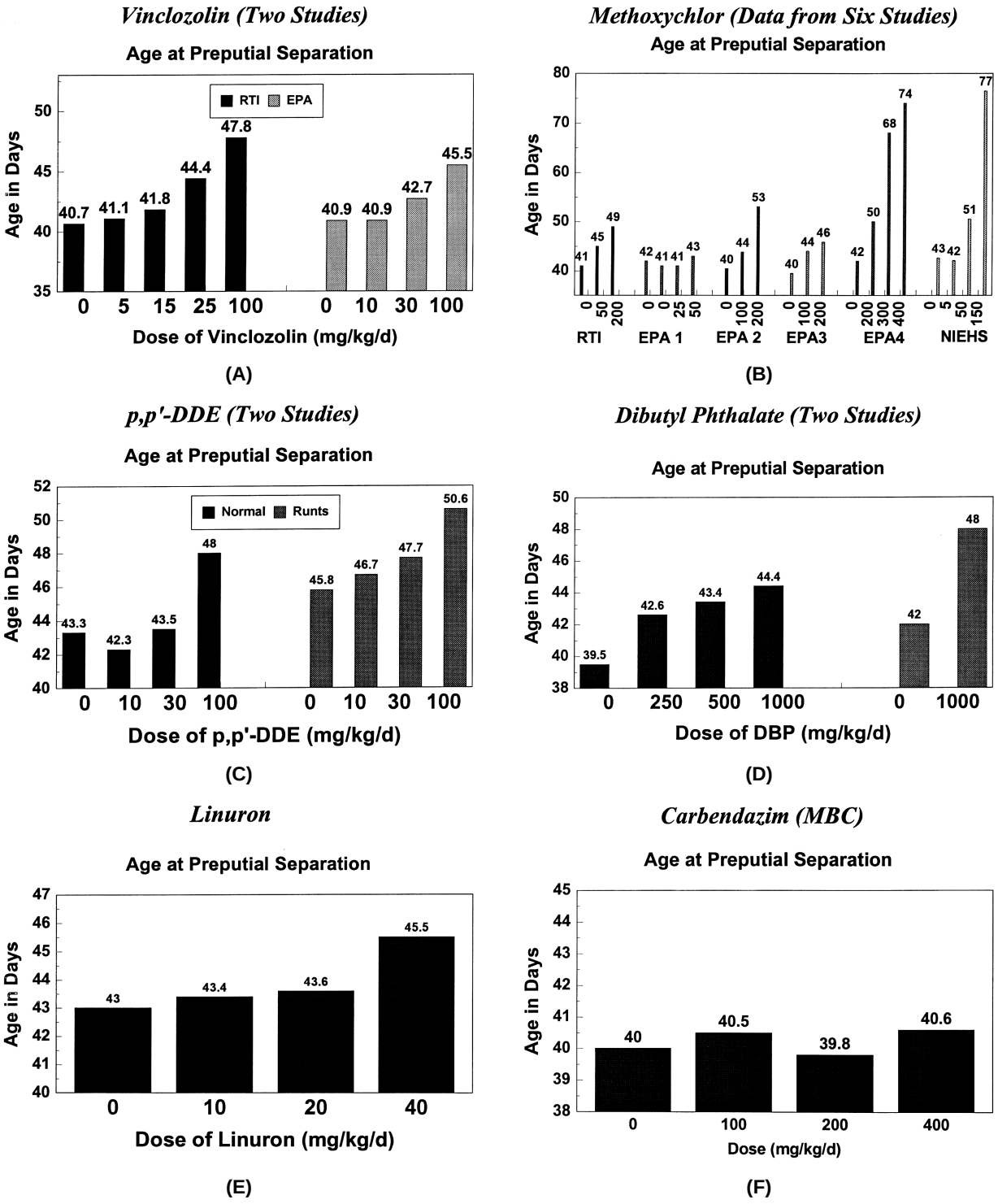


FIGURE 2. Age at preputial separation (PPS) with prepubertal or throughout puberty exposure to five antiandrogenic compounds and one nonendocrine-mediated toxicant. (A) Interlaboratory comparison of the effect of vinclozolin treatment (0 to 100 mg/kg/d) on PPS between the U.S. EPA and the Research Triangle Institute (RTI). (B) Interlaboratory comparison and replicate studies of the effect of methoxychlor (0 to 400 mg/kg/d) on PPS. One data set is from RTI, one from the National Institute of Environmental Health Sciences and four from the U.S. EPA. (C) Two replicate studies of the effect of *p,p'*-DDE (0 to 100 mg/kg/d) on PPS conducted at the U.S. EPA comparing the responses of normal and runt animals. (D) Two studies from the U.S. EPA on the effects of dibutyl phthalate (0 to 1000 mg/kg/d) on PPS. (E) The effect of linuron (0 to 40 mg/kg/d) on PPS from the U.S. EPA. (F) The effect of carbendazim (0 to 400 mg/kg/d) on PPS from the U.S. EPA. (Ref. Monosson et al., 1999).

lase (catabolizing progesterone to 17α-hydroxyprogesterone), and 17–20 lyase (catabolizing 17α-hydroxyprogesterone to androstenedione), but conversely showed increased activities for 17α-dehydrogenase (catabolizing androstenedione to testosterone) (Foster et al., 1983). These compounds do not, however, appear to act as androgen receptor ligands (Gray et al., 1999; Lambright et al., 1999). For this reason it is important to identify an assay to detect androgenic/antiandrogenic activity that is not solely mediated through the androgen receptor.

D. Prolactin Alterations

Prolactin, growth hormone and LH, with or without androgens, play important roles in the development of the normal prostate (Reiter et al., 1999). Prolactin has a synergistic effect with testosterone in stimulating growth of some of the sex accessory glands, especially the lateral prostate (Kolbusz et al., 1982). For example increasing serum prolactin levels with pituitary grafts for 3 weeks delays the castration-induced regression of the lateral lobe of the prostate in adult Sprague Dawley (SD) rats. However, the weight of the sex accessory sex organs was unaffected by higher prolactin levels (Chang, 1984). When ovine prolactin is administered beginning around PND 20 or an ectopic pituitary is placed underneath the kidney capsule, the resulting increase in serum prolactin levels has also been shown to advance PPS and increase testes, prostate and epididymis weight (Aguilar et al., 1988; Negro-Vilar et al., 1977) and seminal vesicle weight (Coert et al., 1985). The effect of prolactin on the sex accessory tissues was demonstrable in the absence of testicular or adrenal steroids (Negro-Vilar et al., 1977). Conversely, the administration of bromocriptine, a dopamine (DA) agonist, prior to puberty, lowers serum prolactin, decreases circulating androgen levels, and decreases accessory gland development (prostate, seminal vesicles), depending on the time and duration of exposure (Kovacevic et al., 1982; Suescun et al., 1985). Similarly, endogenous opioid peptides have a stimulatory influence on prolactin secretion (Hou and Voogt, 1999). Naloxone, an opioid antagonist, administered from PND 51 to 58 reduced serum prolactin and androgens (Maric et al., 1987). Thus, it appears that prolactin at puberty may synergize with androgens and maintain both androgen receptors (Coert et al., 1985) and LH receptors on the testes (Huhtaniemi et al., 1984).

In addition to the effect of prolactin on sex accessory tissue weights, a recent study has shown that an exposure to pimozide, a DA antagonist, from PND 22 to 32 resulted in an increased weight and incidence of lateral prostate inflammation at 120 days of age (Stoker et al., 1999). Estradiol and bisphenol A exposure in this prepubertal time period also resulted in increased serum prolactin during dosing and lateral prostate inflammation in adulthood, with an increased lateral prostate weight in the bisphenol A group (Stoker et al., 1999).

E. Hypothalamic-Pituitary Axis/ Gonadotropins

The target site of many of the toxicants that affect the age of onset of puberty in the male rat is the brain, or more specifically the hypothalamic regions that effect neural control of the pituitary's release of the gonadotropins (see Table 3). The primary mechanism of action of the majority of these toxicants can be receptor modulation or synthesis of neurotransmitters or other factors involved in the control of pituitary gonadotropin release by GnRH. Alterations in GnRH actions with compounds that are known to disrupt GnRH receptor binding, such as directly by antagonists, or indirectly by alterations in the neural input to the GnRH neurons, can delay puberty in the male rat. Examples of such exposures during various times prior to or during puberty and their subsequent effects on the male's endocrine profile, puberty onset, or reproductive tissues are shown in Table 3. For example, several investigators have examined the effects of GnRH antagonists during the pubertal period. Administration of GnRH antagonists beginning on PND 22 delays PPS in a dose-response fashion. However, effects of this exposure on adult FSH levels, testicular weight, fertility, and sexual behavior are more dependent on age and duration of administration (van den Dungen et al., 1989). In addition, the administration of a GnRH analogue from PND 28 to 41 delayed preputial separation by 1 week and resulted in a decrease in testosterone and the weight of the sex accessory tissues at PND 62 (Wisner et al., 1983), presumably by the down-regulation of pituitary GnRH

receptors and consequent reductions in serum gonadotropin and sex steroid levels. Increases in 5α-reductase and serum FSH at the higher doses were also reported.

Exposure to compounds that disrupt neurotransmitter synthesis has also been shown to alter the release of gonadotropins from the pituitary. For example, α-methyl-ρ-tyrosine (AMPT) decreased serum LH and testosterone, but only if administered on PND 38 or later (Raum and Swerdloff, 1986). In contrast, administration prior to this age resulted in an opposite effect. This effect was suggested to be due to an inhibitory role of norepinephrine and dopamine before puberty and the stimulatory role present at puberty onset (Raum and Swerdloff, 1986).

Because some opiates are commonly abused by a subpopulation of adolescent young men (Johnston et al., 1987) and because the existing evidence suggests that endogenous opioid peptide receptor modulators can affect GnRH release, a number of studies have examined the effects of opioid exposure during puberty in the male rat. A single dose of morphine, an opioid agonist, administered between PND 25 and 60 decreased serum LH levels 3 h later. However, treatment with naloxone, an opioid antagonist, between PND 35 and 58 appears to increase serum LH and decrease serum prolactin (see Table 3; Aubert et al., 1988; Maric et al., 1987). A decrease in serum testosterone and DHT following a single dose of naloxone between PND 51 and 58 was suggested to be due to the drug-induced hypoprolactinemia and a decrease in LH receptors in the testes (Maric et al., 1987; Stojilkovic et al., 1987). However, naltrexone, another opiate antagonist administered on PND 55, increased serum testosterone (Emanuele et al., 1998). Therefore, the reported effects of opioid antagonists on serum testosterone and DHT peripubertally seem to be controversial with respect to androgen production.

Increased ethanol consumption in human teenagers has led to concerns of significant hormonal changes during puberty (Bailey and Rachal, 1993). In rats, acute ethanol administration prior to puberty profoundly decreases serum LH levels, which decreases testosterone secretion (Emanuele et al., 1998; see Table 3). More extended dosing of ethanol via a liquid diet from PND 25 to 63 resulted in decreased serum testosterone and seminal vesicle and testes weights; however, serum LH was elevated (Cicero et al., 1990). This was suggested to be due to the extended period of testosterone suppression and thus a decreased feedback suppression on LH. Also, IGF-I and GH suppression have been reported when ethanol was administered during this pubertal period (Steiner et al., 1997). The suppression of these growth factors, in addition to the suppressed testosterone levels, is important because GH and testosterone both play critical roles in organ maturation during this stage of development.

The effects of tetrahydrocannabinol (or Δ^9 THC, the psychoactive ingredient of marijuana) have also been well studied in the male rat during puberty. Significant effects of Δ^9 THC on the release of the gonadotropins have been reported. These effects have been attibuted to a direct action of Δ^9 THC on the control of gonadotropin release by the pituitary, with a decreased response to stimulation by GnRH administration (Gupta and Elbracht, 1983). One study on THC, which generally followed the EDSTAC male pubertal protocol (dosing from PND 21 to 51), found a decrease in serum LH and GH concentrations and a decrease in prostate weight (Collu et al., 1975). In this study, no differences in seminal vesicle or testes weights were reported. In another study employing a pubertal exposure of THC (i.e., PND16 to 40), the authors reported a decrease in serum testosterone, DHT, LH, and FSH on PND 40. By PND 87, the serum LH had recovered; however, when they dosed from PND 16 to 87, the decrease in serum LH persisted. In addition to the hormonal changes, they also observed a decrease in body weight (Gupta and Elbracht, 1983).

Because glutamate activity increases prior to puberty and is involved in an increased release of GnRH, a glutamate agonist, such as *N*-methyl-D-aspartatic acid could conceivably advance puberty in the male rat; however, this has only been shown in the monkey (Plant et al., 1989) and a glutamate antagonist such as the NMDA receptor antagonist, MK 801, could delay puberty as shown in the female (for review see Brann and Mahesh, 1995). In 30-day-old male rats, it has been shown that NMDA (at 15 mg/kg) stimulated the release of LH 15 min after injection, and MK-801 (0.25 or 0.50 mg/kg) decreased LH levels (Pinilla et al., 1998).

Other toxicants have been shown to have direct effects on the pituitary, such as lithium chlo-

ride (Ghosh et al., 1991). By dosing from PND 35 to 50, 55 or 60 with 2.0 mg/kg/d (males killed following last dose), these investigators observed a decrease in serum LH, FSH, T, and PRL at all three dose periods**.** The continuation of dosing to PND 55 and 60 resulted in a decrease in testes, prostate, and seminal vesicle weights.

Finally, environmental conditions, such as light and the daily melatonin rhythms, can also affect the release of gonadotropins. As discussed previously, injections of melatonin from PND20-40 in the afternoon or late photophase have been shown to decrease GnRH receptors, thus lowering serum LH, T, and FSH, as well as decreasing the weights of the seminal vesicles and testes at 40 days of age. As mentioned earlier, this effect may be due to increases in NPY following melatonin administration, in which a delay preputial separation was observed (Corder et al., 1992).

F. Toxicant Effects on Growth Hormone

Several compounds have been shown to have effects on growth hormone (GH). For example, sodium pentobarbital blocks the release of somatostatin and GRF, and thus suppresses the release of GH from the pituitary (Wehrenberg et al., 1982). During puberty, several toxicants have been shown to alter GH release and IGF-I levels. A decrease in GH is associated with a decrease in IGF-I levels, as mentioned, and it is this IGF-I that also influences the release of GnRH. Therefore, many compounds that suppress GH and, in turn, suppress IGF-I also decrease serum LH levels and androgen-associated endpoints. For example, ethanol administered in a single dose between PND 35 and 51 results in a suppression of GH and/or serum LH and testosterone, depending on the day dosed prior to puberty (see Table 3; Emanuele et al., 1998; Tentler et al., 1997). This profile has also been seen following a 30-day exposure to THC from PND 21 to 51 (similar to our pubertal protocol with sacrifice at 51 days), with a decrease in GH and serum LH (Collu et al., 1975). This exposure to THC also decreased the weight of the prostate, which may be due to a decrease in androgen production as seen with other exposures during puberty associated with the decrease in serum LH (Gupta and Elbracht, 1983).

This feedback regulation of GH and IGF-I has also been shown using GH antibodies and GH analogues. For example, the administration of antibodies against rat GH-releasing factor (GHRF) between PND 15 and 39 in male rats resulted in a decrease in IGF-I, decreased testes and seminal vesicle weight, and decreased sFSH (Arsenijevic et al., 1989). They attributed the decreased weights to the low IGF-I levels.

G. Toxicant Effects on Thyroid Function

Certain drugs inhibit thyroid function by antagonizing the formation of thyroid hormones. These drugs are typically separated into the drugs that inhibit iodide transport (iodide trapping) into the thyroid gland and those that inhibit the incorporation of iodine into tyrosine. Inhibitors of iodide transport include thiocyanate and other monovalent anions like perchlorate, chlorate, and periodate. Other antithyroid compounds include the thionamides, the sulfonamides (e.g., para-aminobenzoic acid), and the sulfonylureas (carbutamide and tolbutamide). An effective concentration of these drugs for a sufficient time will result in thyroid hypertrophy and goiter, so they are often referred to as “goitrogens”. Clinically, these drugs are used to treat excessive thyroid hormone production (Hadley, 1996).

Another group of goitrogenic chemicals disrupt thyroid hormone homeostasis by increasing the peripheral catabolism of the thyroid hormones through an induction of hepatic microsomal enzymes. These include calcium channel blockers, steroids, retinoids, chlorinated hydrocarbons, polyhalogenated biphenyls, and enzyme inducers (Capen, 1997). Some chemicals disrupt thyroid function by inhibiting thyroperoxidase (TPO), an enzyme involved in the oxidation of iodide to its active form in the synthesis of thyroid hormones, such as sulfamethazine (antibacterial compound used in food processing), thiourea, propylthiouracil, sulfonamides, methimazole, aminotriazole, and acetoacetamide (Capen, 1998).

Environmental chemicals can alter thyroid function by altering synthesis, release, transport, or metabolism of thyroid hormones or by potentially acting as receptor agonists or antagonists, although none identified. (Capen, 1997). Several environmental agents have been found to alter thyroid hormone levels, such as urea derivatives, polyhalogenated biphenyls, and chlorinated dibenzo-*p*-dioxins. Propylthiouracil (PTU) and me-

thimazole have been shown to result in decreased T4 and T3. Other chemicals that induce hypothyroidism, such as PCB, TCDD, methoxychlor, thiocarbamide, and sulfonamide-based pesticides, reduce circulating thyroid hormones, elevate TSH and result in thyroid follicular neoplasia (Crisp et al., 1998). Some compounds may also have effects on hepatic enzyme-inducing drugs that clear thyroid hormones from the circulation and thus elevate TSH. For example, if the chemical induces cytochrome P-450s and increases the metabolism of the thyroid hormones, TSH becomes elevated in response to reduced circulating levels of T3 and T4. Toxicant-induced alterations in thyroid hormones during puberty could have permanent effects because these hormones are critical to normal growth and development. The endpoints to be used in the male pubertal protocol for thyroid assessment are thyroid hormone levels (TSH and T4 for thyroid hormone homeostasis) and histopathology of the thyroid gland. Thyroid gland histopathology has been determined to be the most reliable parameter for the detection of compounds that affect thyroid function (DeVito et al., 1999). Shrinkage of the thyroid follicle colloid area and increased cell height is consistent with the movement of stored thyroid hormones from the colloid region stimulated by TSH in response to low circulating levels of T4 (Capen, 1997). There is concern whether a short dosing period, as in the pubertal protocol, is long enough to detect weak-acting chemicals that target the thyroid gland (O'Connor et al., 1998). However, Biegel et al., 1995, found that 2 weeks is sufficient for some chemical changes of the thyroid gland. The typical hormonal pattern for compounds that alter thyroid function in rodents is decreased serum levels of T3 and T4 and increased serum TSH levels, in which the sustained release of TSH results in follicular cell hypertrophy/hyperplasia.

Hypothyroidism induced by toxicant exposures during the early neonatal period, and its effects on reproductive endpoints have been well characterized. For example, the induction of hypothyroidism by the administration of PTU or polychlorinated biphenyls from birth to weaning increased testis size, number of Sertoli cells and Leydig cells, and daily sperm production in the adult (Cooke and Meisami, 1991; Cooke et al., 1996; Hardy et al., 1996). Similar reports show that the critical period for this effect is the first 2 weeks after birth (Cooke et al., 1992; Meisami et al., 1992), with no effects observed if PTU treatment was started during late lactation. Other studies show that the induction of hypothyroidism by administration of methimazole from birth to weaning caused a delay of testis growth at 3 to 4 weeks of age (Palmero et al., 1994). These differences are believed to be due the retardation of Sertoli cell differentiation up until day 30, with an increase above controls beginning on PND 36, leading ultimately to increased testis size in the adults (Van Haaster et al., 1992). In addition, inducing hypothyroidism with methimazole treatment after birth resulted in reduced levels of gonadotrophins and a delay in pubertal spermatogeneis by retarded differentiation of the Sertoli cells (Francavilla et al., 1991).

There are relatively few examples of studies evaluating toxicant-induced alterations in thyroid hormones during the peripubertal period in the male. However, Ching (1981) did report that a dose of barbital (diethylbarbituric acid) or PTU to male rats from PND 28 to 56 resulted in an increased level of serum TSH and thyroid weight and a decreased level of thyroid hormone (T4). This effect of barbital has been shown to be due to hepatic microsomal changes (i.e., induction) in cytochrome P-450 isoenzymes (McClain et al., 1988), which increase catabolism of the thyroid hormones.

In addition to altering several aspects of growth and development, altered thyroid hormone function during the prepubertal period can dramatically impact reproductive development. For example, an induction of hypothyroidism by administration of methimazole beginning on day 40 decreased serum LH, PRL, T, T3, and T4 at 100 days of age (Valle et al., 1985).

VIII. TECHNICAL CONSIDERATIONS IN THE CONDUCT OF THE MALE PUBERTAL SCREENING

The primary purpose of the male pubertal protocol is as a first-tier screen for endocrine active chemicals by determining whether a chemical exposure can alter the development of the reproductive system and the thyroid gland. The literature reviewed in the preceeding sections using PPS as an

endpoint indicated that this assay is promising; however, a rigorous evaluation of the specific male pubertal protocol must be completed before any recommendations can be made. If followed properly, the protocol should produce data that are consistent across different laboratories and over a broad range of compounds. In this regard, this protocol has been shown to be a robust and sensitive tool in the evaluation of several endocrine-disrupting chemicals. However, there are several technical concerns that need to be addressed for the use of the male pubertal assay. In this regard, the following concerns should be considered, in light of the literature review presented here, and used to reduce the variability and to assist in the interpretation of the data.

The companion manuscript on the evaluation of female pubertal development also discusses several issues that are also of concern for conducting the pubertal assay in the male, including issues such as the diet, interpreting body weight changes, and hormonal assessments. The importance of these issues to the male, as well as the female, protocol is obvious. However, there are also a number of technical issues that are specific to the male protocol (Table 1), which are addressed in the following sections.

A. Purpose and Applicability

The purpose of this assay is to detect the effects of environmental compounds on pubertal development and thyroid function in the intact male rat. There are several generic issues concerning the assessment of the pubertal animals that will be discussed as they relate to the required and optional endpoint measures.

B. Endpoints

The protocol lists several required and optional endpoints, including tissue weights, hormonal measures, and histological evaluations. With the required endpoints, observations can be made concerning the effects of endocrine disruptors on growth, PPS, and the weight of androgen-responsive tissues on the final day of dosing. At necropsy, the paired testicular, paired epididymal, ventral prostate (VP), seminal vesicle (SV; with coagulating glands and fluid), levator ani plus bulbocavernosus muscles (LABC), and body weights are to be recorded. The epididymides, ventral prostate, seminal vesicles (SV), and levator ani are all androgen-sensitive tissues that show reduced size following exposure to antiandrogens (e.g., see Table 4). Depending on the mechanism of antiandrogen action (receptor mediated vs. inhibition of steroidogenesis) these organs may, however, respond differentially. For example, in the case of AR antagonists, differences in the responsiveness of the LABC vs. the sex accessory glands have been noted. While 5-α-reductase inhibitors reduce sex accessory gland size typically with less effect on LABC weight, androgen receptor antagonists reduce both accessory gland size and LABC weight.

In addition to required endpoints that evaluate androgen-responsive tissues, the protocol also includes endpoints to examine alterations of thyroid function (as determined by T4, TSH, and thyroid histology). These thyroid endpoints seem appropriate to identify alterations in thyroid function, as compounds that affect thyroid hormone homeostasis can lead to a decrease in T4 and T3 and a subsequent sustained release of TSH, which then leads to follicular cell hypertrophy/hyperplasia (Table 4). At the 1997 thyroid workshop entitled “Screening Methods for Chemicals That Alter Thyroid Hormone Action, Function and Homeostasis”, thyroid gland histopathology was determined to be the most reliable parameter for the detection of compounds that affect thyroid function (DeVito et al., 1999). As mentioned previously, 2 weeks is sufficient for some chemical changes of the thyroid gland; however, a 30-day exposure is needed to detect weak-acting chemicals that target the thyroid gland (O’Connor et al., 1998).

Additional information would be obtained from the optional endpoints to more accurately assess the possible mechanism of action of the environmental chemicals (see Table 4 for summary of effects on hormonal endpoints); however, these measures are not necessary for an initial screen in confirming an effect. For example, testosterone levels would be useful for identifying an effect; however, it would be a very nonspecific measure. Therefore, the response of the weights of tissues that are responsive to androgens was chosen to more easily and accurately identify effects of steroidogenic compounds.

C. General Conditions

Some laboratory rodent diets contain phytoestrogens that could potentially interfere with identifying effects on the progression of male puberty (Casanova et al., 1999; Thigpen et al., 1999), since as discussed previously, estrogen exposure to the pubertal male can result in delays in puberty and can also inhibit food consumption. Thus, a low phytoestrogen-containing diet should be used in conducting this protocol and kept consistent across laboratories to reduce the possible interference of estogenic effects.

D. Animals

The current protocol uses Sprague-Dawley or Long Evans hooded rats derived from timed pregnant females, which are suggested to arrive on gestation day 7 to 10. These females should arrive during early gestation (after implantation or GD6) to prevent exposure to dietary phytoestrogens during mid to late gestation, which could have subsequent effects on the development of the offspring (Casanova et al., 1999). Also, this source of timed pregnant females should be required in order for weaning and weight ranking to be completed prior to first day of dosing on PND 23.

It seems that if interlaboratory variability is to be minimized, the protocol should only use one strain of rats to identify the effects of environmental toxicants on pubertal progression and thyroid function. Although the average age of preputial separation is similar between these two strains, there are likely differences in other parameters such as metabolism, distribution, and body weight that might make one strain more sensitive than another to a chemical. However, the use of two strains could identify the broader effects of some endocrine-disrupting chemicals, depending on strain selection.

The protocol states that litters should be culled to 8 to 10 on PND 3 or 4. Our laboratory has observed a greater survival rate of pups, culled on PND 4 and in addition allows more time for runts to be identified. In this regard, the protocol should recommend a specific litter size (either 8, 9, or 10) to aid in reducing reducing the variability in milk consumption and subsequent body weight at PND 21.

In addressing the issue of culling the litters on PND 3 or 4, one of our laboratories has found that randomly standardizing litters to eight pups vs. keeping them in natural litter sizes enhances growth and reduces the variation in body weight at weaning and does not alter the age at puberty in the male or female Long Evans Hooded (LE) rat. In this study 15 litters were standardized (S) and another 15 retained natural (N) litter sizes. Litters were not culled, but rather litter sizes in the S groups were randomly reduced at 4 days of age to to eight pups (four males and four females, where possible). After standardization at 4 days of age, pup weights were 11.8 ± 0.49 grams and 11.4 ± 0.48 g for the S vs. N, respectively. At 23 days of age the male (M) and female (F) rats weighed significantly more than N rats did (SM = 71.5, NM = 64.7, SF = 67.2, NF = 61.2; $p < 0.01$). In addition, S animals had less variable body weight at weaning than did N rats (variances are SF = 17, NF = 36.3, SM = 27, NM = 38). The body weight at 23 days of age was not correlated with the age at vaginal opening ($r = -0.05$) or preputial separation ($r = -0.04$), and the age at puberty did not differ between the two groups. Age at vaginal opening was 32.1 ± 1.34 for the S females vs. 32.8 ± 2.14 for females from N litters and the age at preputial separation was 42.2 ± 3.16 for SM vs. 42.2 ± 2.01 for NM rats. When the males were necropsied at about 70 days of age, body ($SM = 433 \pm 5.8$, $NM = 422 \pm 6.0$), testis ($SM = 3.58 \pm 1.1$, $NM = 3.55 \pm 0.04$) and seminal vesicle ($SM = 1.11 \pm 0.04$, $NM = 1.08 \pm 0.03$) weights did not differ between the two groups.

On PND 21 the population of males are selected by eliminating the weight outliers with a range of 8 g above and below the mean. This range was determined by historical weanling male rats in our laboratory as approximately 2 standard deviations from the mean. Studies that used runted litters in this protocol still observed effects on pubertal progression, despite the reduced body weight at weaning. For example, in one of our studies examining the effects of *p,p′* DDE (Figure 2c), the "runted" block of males weighed about 50 g at 25 days of age vs. about 75 g in the "normal" study. *p,p′* DDE administration at 100 mg/kg/d to pups of normal weight delayed the age of PPS by about 5 days ($p < 0.0001$), while the runted animals attained puberty about 2.5 days later ($p < 0.0001$, no interaction, $p > 0.5$). *p,p′* DDE-

treated animals weighed almost 40 g more at puberty than did controls, while the runted animals generally weighed 30 g less than the "normal" male rats at equivalent dosage levels. These data are just one example that contribute to the proposal that puberty is not attained at a "critical" body weight, and that chemicals can delay this process without retarding general somatic growth. However, as discussed previously, the contribution of other factors, such as percentage of body fat and protein and the secretion of leptin, have also been shown to influence puberty onset. In this regard, the role of body weight and metabolic status on the timing of puberty cannot be dismissed.

E. Experimental Design

Varying dosage levels of the xenobiotic can be tested, with one required high-dosage level at or just below the maximum tolerated dose (MTD), which is typically described as the dose level at which the treated animals have a body weight gain that is 10% below the controls, in which the animal has no clinical signs of toxicity. The route of exposure of the reported MTD and the rat strain need to be carefully considered when selecting this dose. As mentioned, an estrogenic compound could result in a decrease in food consumption (Reynolds and Bryson, 1974), so the limitation of a 10% reduction in body weight for the required high dose could mask the effects of some chemicals if the dose that lowers body weight is lower than that which causes other reproductive effects (see earlier discussion in Leptin/Growth section).

F. Treatment

Initially, the pubertal male assay, published in the Final EDSTAC Report, proposed to expose rats orally to the xenobiotic for 20 days, from 33 to 53 days of age. However, this assay has since been modified and the dosing period expanded from PND 23 to 53. The change was adopted to provide better coverage of the entire peripubertal period and to increase the sensitivity to thyroid alterations. Although, as mentioned, thyroid histology alterations can be observed as soon as 2 weeks, most studies of thyroid function are at least 28 days in duration (DeVito et al., 1999). In addition, dosing now includes 1 week prior to the decline in testicular 5α-reductase activity, the enzyme that is responsible for a switch from 3α-diol secretion by the testes to the more potent androgen, testosterone (Figure 1a). Continuing dosing until 53 days of age was deemed desirable because it incorporates the period during which the seminal vesicles begin to accumulate fluid in response to continued testicular testosterone secretion (Figure 1a and d) and sperm from the maturing testes first appear in the caput and corpus epididymide. Even a slight delay in the onset of puberty should result in a reduction in seminal vesicle weight and epididymal sperm numbers at this age (e.g., Monosson et al., 1999).

The route of exposure will affect the results for some chemicals, because the pharmacokinetics vary significantly between oral, intraperitoneal, and subcutaneous routes. For example, bisphenol A will have less estrogenic activity by the oral route, as its bioavailability is very low following oral exposure (Potterger et al., 1998) when compared with the more effective subcutaneous route (Ashby and Tinwell, 1998). The opposite is true for methoxychlor, which is known to be more effective by oral gavage, because it is metabolized to the estrogenic form, HPTE, in the liver (Bulger et al., 1978).

The time of day of dose administration is also important because some hormones have a 24 h rhythm associated with room lighting. Therefore, the time of the daily treatment may affect a critical period or time of increased secretion for certain hormones, depending on the pharmacokinetics of the chemical. Thus, to minimize this variable, the protocol states that the animals should be dosed within a restricted time period (0700 to 0900 h). On the day of necropsy, the time of dose is especially important because the concentration of the test compound in the serum and tissue can have effects on specific hormonal endpoints.

G. PPS

Preputial separation is a reliable noninvasive indicator of the androgen status of the pubertal rat (Gaytan et al., 1988), and it is easy to observe the day of complete cleavage of the epithelium lining

the prepuce of the penis. As mentioned in the protocol, any additional observations, such as a persistent thread between the glans and the prepuce, should be recorded. PPS normally occurs between 40 and 43 days of age in LE and SD rats. The protocol states that males should be examined daily for PPS. Also, it is important that the males be examined at approximately the same time of day to be consistent between groups with the progression of PPS, and it is also important that the evaluations be conducted blind to treatment.

H. Necropsy

The protocol indicates that the males are necropsied on day 53, but no indication is given about the time of day. Necropsies are generally done with little regard for time of day; however, there are several reasons that there should be some effort to keep the time within a narrow window: time since last dose administered and the presence of daily circadian rhythyms of serum homone secretion or cyclicity of endogeonous hormone measures. For example, there appears to be a circadian rhythm in the secretion of TSH in the rat. There is a zenith in serum TSH levels at midday (1130 ± 1 h) (Jordan et al., 1987; Pallardo et al., 1976; Selgas et al., 1997), with a minimum period of TSH secretion at the beginning of the dark period. Peaks in the secretion of T3 and T4 typically occur 1 to 2 h following the TSH elevation, with a more narrowly defined window for T3 than for T4 (Jordan et al., 1980). In addition, prolactin is lowest in the morning hours 4 h after lights on, with an increase about 1 h before lights out in a 12:12 light schedule (Ramaley, 1981). Thus, a restricted time period for the necropsy during a period of basal secretion would assure more consistent results between laboratories.

If the intent is to measure LH, testosterone, and/or prolactin or other hormones that are directly affected by acute stress, then the animals must be killed in a manner that avoids stressing the animals. This would include killing the animals quickly by decapitation in a room separate from the home cage. Also, to reduce discrepancies in the hormone data between labs, other environmental stressors present at the time of blood collection must also be minimized. For example, if the animals are to be moved from an animal facility to a new location for the necropsy, the animals should be moved the day before for acclimation to the new holding area. As mentioned, this holding area must be separate from the necropsy area.

At necropsy, the paired testicular, paired epididymal, ventral prostate, seminal vesicle with coagulating glands and fluid, levator ani plus bulbocavernosus muscles, and body weights should be recorded. During necropsy care must be taken to remove mesenteric fat with small surgical iris scissors from these tissues such that the fluid in the sex accessory glands is retained. As indicated earlier, at this age the fluid compartment of the SV is especially dynamic in this androgen-sensitive tissue (Figure 1d).

In addition, care needs to be taken when decapitating so that the thyroid gland is undisturbed for removal (i.e., typically retained with head). Also, because of the difficulty of separating the thyroid from the trachea, the thyroid gland with attached trachea can be removed and placed in the Bouin's fixative and separated from the trachea after fixation for subsequent hisological examination (DeVito et al., 1999).

I. Hormone Measures

While serum T4 and TSH should be measured, serum LH, testosterone, estradiol, T3 and prolactin levels were listed as optional. The primary use of these optional measures would be to serve as adjunct endpoints that could assist in clarifying the mode of action of the test compound. Serum should be stored in siliconized tubes for subsequent radioimmunoassay, which will prevent steroids from adhering to the tube wall.

In performing radioimmunoassays, commercially available steroid assay kits or assays for measuring pituitary peptide hormones using materials provided by the National Institute for Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDKD) are widely used in endocrine assessments. However, the NIADDKD materials are only available to government, academic, or non-profit agencies. If other commercially available kits are to be used for the pituitary peptide hormones, any discrepancies between assays need to be considered in interpretation of data.

Chemical treatments can increase the Coefficient of Variation (CV) for T and LH, resulting in

less statistical power in the analysis than one would anticipate based on the control values. Despite the inherent variability in these hormones, treatments with antiandrogens, like Vinclozolin (which induces a twofold increase in serum LH and T), can produce statistically significant alterations in LH and T. In fact, analysis of log transformed serum LH data indicate that this endpoint was affected at the lowest dosage level used in one study (10 mg/kg/d; Monosson et al., 1999).

J. Statistical Analysis

The EDSTAC protocol states that enough litters should be used to assure that about 45 male pups are available at weaning (for one control and two treatments groups of 15). If more treatment or dosage groups are included, then the numbers of litters and male pups should be adjusted accordingly. In addition, body weight at weaning is also statistically controlled by assigning males to treatment groups in a manner that provides each group with similar means and variances in weaning weight. The magnitude of this source of variance is determined by including weaning weight as a covariate in the data analysis.

All data (age at PPS, weight at PPS, body and organ weights at necropsy, and serum hormones) are analyzed using body weight at weaning as a covariate. If the study was conducted as two blocks, then the analysis is a two-way ANOVA with Block and Treatment as main effects, again, wtih body weight as a covariate. For organ weight data, body weight at necropsy could be used as a covariate in the model. If treatment reduces growth and delays PPS, the mechanism responsible for the delay is always in question. In this regard, body weight change from day 22 until the average age of PPS in the control group could be used as a covariate in the regression model.

Data should be summarized and the mean, SE, and CV values for the control data should be examined to determine if they meet acceptable quality assurance criteria for consistency with normal values. Examples of control and high-dose data from a study on the effects vinclozolin administration are provided in Table 5. Summary data tables should include at least age and weight at PPS, testicular, epididymal, ventral prostate, seminal vesicle (with coagulating glands and fluid), levator ani, liver, and body weights at necropsy, body weight change from day 23 to necropsy, and serum T4 and TSH. Data may be also be presented after covariance adjustment for body weight, but this should not replace presentation of the unadjusted data.

K. Hershberger vs. Pubertal Male Assay

The Hershberger assay, which is to be used in the Tier I screen (T1S), is conducted in PND 22 castrated males, in which the test compound is generally administered from PND 29 to 35 or 38. It is a very sensitive assay for the detection of chemicals that alter AR function. One advantage of the pubertal male assay is that it allows for a comprehensive assessment of the endocrine system. It also allows for an interpretation of the observed endocrine effects in the context of other possible toxic effects of the chemical. This battery of endpoints avoids the pitfall of relying on a single endpoint. Hence, this assay has broader application than the Hershberger because it detects chemicals that affect the hypothalamic-pituitary axis (Hostetter and Piacsek, 1977; Ramaley and Phares, 1983), or inhibit steroid hormone synthesis, as well as chemicals that alter AR function. The major limitation of this assay is that, unlike the Hershberger assay, which takes 7 to 10 days, the pubertal male assay is 30 days long. In this regard, if the pubertal male assay replaced the Hershberger assay in the T1S, then the *in vitro* steroidogenesis assay could likely be dropped as well.

L. Limitations of the Pubertal Male Assay

Although preputial separation, sex accessory gland weights and thyroid hormone analyses and thyroid histology are sensitive to endocrine modulation, a delay in preputial separation is not pathognomonic for antiandrogens. Pubertal alterations also result from chemicals that disrupt hypothalamic-pituitary function (Huhtaniemi et al., 1986), and, for this reason, additional *in vivo* and *in vitro* tests would be needed to identify the mechanism of action responsible for the pubertal alterations. As mentioned, alterations of prolactin, growth hormone, gonadotrophin (LH and FSH) secretion, or hypothalamic lesions can also alter the

TABLE 5
Pubertal and Necropsy data from LE male rats dosed with oil or Vinclozolin (100 mg/kg/d) by gavage from Monosson et al., 1999

	Control males mean/CV	Vinclozolin-treated males
Age at PPS	40.9 days/4.7%	**45.5/5.8%**
Body weight at PPS	201 g/10.2%	**232/11.1%**
Body weight at Necropsy	313 g/7.1%	303/5.3%
Pituitary weight	9.4 mg/ 4.0%	9.7/13.2%
Adrenal weights	50.1 mg/13.2%	**63.5/18.1%**
Kidney weights	2.68 g/11.3%	2.67/7.0%
Liver weight	14.1 g/10.3%	15.3/8.6%
Seminal vesicle and coagulating gland weight (with fluid)	774 mg/16.2%	**550/20%**
SVCG weight (without fluid)	368 mg/14.6%	**287/19%**
Ventral prostate	236 mg/12.2%	**188/12.7%**
Testes weight	2.88 g/5.6%	2.90/10.0%
Testis spermatid count	156 million/9.8%	156/16.3%
Left epididymides	260 mg/7.9%	**229/9.5%**
Epididymal sperm numbers	63.5 million/11.0%	**55.0/12.0%**
Serum testosterone (ng/ml)	2.61/33%	**4.89/49%**
Serum DHT (ng/ml)	0.145/111%	0.184/94%
Serum 3-α-Diol (ng/ml)	0.51/29%	**1.04/38%**
Serum LH (ng/ml)	0.618/34.5%	**2.30/69%**

Note: Dosing was initiated at 22 days of age and continued until necropsy at 51 to 52 days. Values are means and CVs for the control and high dose group V-treated animals. Note the relatively large CVs for serum hormone values, as compared to organ weight data, in control animals and how V-treatment increases the CV. Values in boldface differ significantly from control.

rate of pubertal maturation in weanling rats. The limitations of the pubertal male assay are that (1) it uses live animals, (2) it is apical and the mechanism of action may not be apparent without follow up studies, (3) it is 30 days in duration, and (4) the sensitivity of PPS to antiandrogens that inhibit steroidogenesis has not been well defined. Furthermore, although this assay has been used for experimental purposes for nearly a decade, it has not been thoroughly standardized and validated. In this regard, the EPA recently has begun the validation process for the pubertal male and female assays. In contrast, the Hershberger and uterotropic assays have been used for screening and testing for hormonal activity since the mid-1930s, with standard methods being adopted by official organizations after an interlaboratory validation in the 1960s. Additionally, the pubertal male assay is less sensitive than the Hershberger assay to AR ligands because it uses intact males, whereas the Hershberger assay uses castrated immature rats.

M. Advantages of the Pubertal Male Assay

Some of the advantages of this assay include (1) it does not require surgical manipulation of the animals, (2) the endpoints are sensitive to chemical perturbation, (3) most of the end points already are part of the USEPA Multigenerational Harmonized Test Guidelines (organ weights, histology,

and PPS) or have been used for screening for endocrine activity in rats for decades (T4, TSH, thyroid histology) so the assay should be relatively easy to implement. As mentioned previously, the pubertal male assay has broader utility than the Hershberger.

Another advantage of the pubertal male assay is that it allows for comprehensive assessment of the endocrine system. It is apparent that PPS can be more sensitive to some AR antagonists than are organ weights or altered HPG function, while in other cases the reverse is true (Gray et al., 1999; Kelce et al., 1995). In studies of vinclozolin, increases in serum LH were a sensitive response to this antiandrogen, whereas serum LH is not increased in males exposed to *p,p′* DDE during puberty (Kelce et al., 1995). Hence, one cannot rely on a single endpoint but rather a battery of endpoints should be retained in the assay, as described. Similar to the pubertal male assay, the pubertal female assay (a T1S assay) also is sensitive to alterations of the HPG axis and steroidogenesis (including aromatase). While the pubertal female assay cannot reliably detect AR agonists and antagonists, puberty (VO) in the female is more sensitive to xenoestrogens than it is in male rat (PPS). For example, dietary administration of nonylphenol affects VO, but not PPS (Chapin et al., 1998) and estradiol (Biegel et al., 1998) and methoxychlor (Chapin et al., 1997; Gray et al., 1989), accelerate VO at dosage levels nearly fivefold below those that delay PPS. Some expected effects of the pubertal male protocol are shown in Table 6.

TABLE 6
Pubertal Male Assay Response/Weight of Evidence Table

Endpoint	AR antagonist Type 1	Strong ER agonist, AR antagonist	AR antagonist Type 2	5α-reductase inhibitor	PCB	P450 Inhibitor
Growth	–	++	–	–	–	–
Age at preputial separation	++	+	++	++	–	+
Weight at PPS	++	+	++	++	–	+
Serum T4	–	–	–	–	+	–
Serum TSH	–	–	–	–	– +	–
Seminal vesicle weight	++	+	–	+	–	+
Testis Weight	+	+	–	–	–	+
Thyroid histology	–	–	–	+	–	–
Ventral prostate weight	++	+	–	+	–	+
Levator ani plus bulbocavernosus weight	++	+	–	–	–	+
Nonreproductive organ weights	**Liver ↑ adrenal**	+	**Liver**	–	**Liver**	–
Weight of evidence decision: for this assay	Positive	Weak Positive	Positive	Positive	Positive	Positive

Note: Known or expected responses of well characterized toxicants in the Pubertal Male Assay. The list includes only required endpoints. Responses are indicated as: + Is positive, – is negative, + –?, is a possible positive response, and shaded responses are more certain than unshaded ones. A weight of evidence decision is provided as: positive or negative for EAT activities.

TABLE 6 (continued)
Pubertal Male Assay Response/Weight of Evidence Table

Endpoint	Thyroid mimetic	Aromatase inhibitor	Potent inhibitor of T4 synthesis	Phthalate	Anti-ER	Weak ER ligand
Growth	–	–	–+	–	–	–
Age at preputial separation	–	–	–+	++	–	–
Weight at PPS	–	–	–+	++	–	–
Serum T4	+	–	++	–	–	–
Serum TSH	+	–	++	–	–	–
Seminal vesicle weight	–	–	+–	++	–	–
Testis weight	–	–	+–	++	–	–
Thyroid histology	–	–	++	–	–	–
Ventral prostate weight	–	–	+–	++	–	–
Levator ani plus bulbocavernosus weight	–	–	+–	++	–	–
Nonreproductive organ weights	–	–	–	–	–	–
Weight of evidence decision: for this assay	Positive	Negative	Positive	Positive	Negative	Negative

N. Reproducibility of the Pubertal Male Assay

An important consideration in the evaluation of an assay for screening for endocrine activity is how reproducible the results are from study to study and from laboratory to laboratory. It is also important to detemine if the effects are"robust" (i.e. minor protocol variations do not compromise the responses). In this regard, the effects of several toxicants on the timing of PPS have been replicated successfully by several laboratories. As indicated earlier, the ability of vinclozolin to delay PPS at 100 mg/kg/d has been demonstrated in two laboratories by different investigators (Anderson et al., 1995; Monosson et al., 1999; Figure 2a). In addition, the effects of vinclozolin on serum LH and T also were replicated in two different laboratories (Anderson et al., 1995), even though the age at necropsy differed considerably between the studies (55 days of age vs. adults). In addition to vinclozolin, in six separate experiments from three laboratories, methoxychlor treatment, through the pubertal period delayed PPS at similar dosage levels (Figure 2b). Taken together, these results indicate that effects of these toxicants on PPS are reproducible and robust. Furthermore, some testicular toxicants, such as carbendazim, can have no effect on PPS, despite its effects on spermatogenesis. Therefore, such testicular toxicants that do not alter endocrine system functioning or affect other factors that may also be involved in pubertal progression in the male may not delay PPS (Figure 2f).

IX. SUMMARY

The existing data indicate that this assessment of pubertal development and thryoid function in

the male rat is a simple and effective method to detect the endocrine activity of pesticides and toxic substances. As indicated above, such a simple and sensitive assay for EDCs may provide some advantages over other methods under consideration that utilize castrate-hormone–treated males (Endocrine Disrupters Screening and Testing Advisory Committee [EDSTAC], 1998). As reviewed here, toxicants administered from 23 to 53 days of age in the "male pubertal assay" successfully identify the endocrine activity of several antiandrogenic and estrogenic pesticides. The scientific basis for the longer duration of the study, when compared with the shortened versions proposed by Ashby and Lefevre (1997) and in the proposed EDSTAC protocols (Final Report, 1998) is also provided.

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REFERENCES

Aguilar E, Tena-Sempere M, Gonzalez D, and Pinilla L (1996). Control of gonadotropin secretion in prepubertal male rats by excitatory amino acids. *Andrologia* **28:** 163–9.

Aguilar R, Bellido C, Sanchez-Criado JE, and Aguilar E (1988). Mechanisms of precocious puberty induced in male rats by pituitary grafts. *J Reprod Fertil* **83:** 879–83.

Anderson SA, Pearce SW, Fail PA, McTaggart BT, Tyle RW, and Gray LE Jr (1995). Validation of the alternative reproductive test protocol (art) to assess toxicity of methoxychlor in rats. *The Toxicologist* **15**.

Aragona C, Bohnet HG, and Friesen HG (1977). Localization of prolactin binding in prostate and testis: The role of serum prolactin concentration on the testicular LH receptor. *Acta Endocrinol (Copenhagen)* **84:** 402–9.

Aragona C and Friesen HG (1975). Specific prolactin binding sites in the prostate and testis of rats. *Endocrinology* **97:** 677–84.

Arendt J (1997). Safety of melatonin in long-term use (?). *J Biol Rhythms* **12:** 673–81.

Arsenijevic Y, Wehrenberg WB, Conz A, Eshkol A, Sizonenko PC, and Aubert ML (1989). Growth hormone (GH) deprivation induced by passive immunization against rat GH-releasing factor delays sexual maturation in the male rat. *Endocrinology* **124:** 3050–9.

Ashby J and Lefevre PA (1997). The weaning male rat as an assay for endocrine disruption: preliminary observations. *Reg Toxicol Pharmacol* **26:** 330–337.

Ashby J and Tinwell H (1998). Uterotrophic activity of bisphenol A in the immature rat. *Environ Health Perspect* **106:** 719–20.

Aubert ML, Begeot M, Winiger BP, Morel G, Sizonenko PC, and Dubois PM (1985). Ontogeny of hypothalamic luteinizing hormone-releasing hormone (GnRH) and pituitary GnRH receptors in fetal and neonatal rats. *Endocrinology* **116:** 1565–76.

Aubert ML, Rivest RW, Lang U, Winiger BP, and Sizonenko PC (1988). Delayed sexual maturation induced by daily melatonin administration eliminates the LH response to naloxone despite normal responsiveness to GnRH in juvenile male rats. *Neuroendocrinology* **48:** 72–80.

Bailey SL and Rachal JV (1993). Dimensions of adolescent problem drinking. *J Stud Alcohol* **54:** 555–65.

Ball P, Emons G, and Gethmann U (1981). Effect of low doses of continuously administered catecholoestrogens on peripheral and central target organs. *Acta Endocrinol (Copenhagen)* **96:** 470–4.

Baranao JL, Tesone M, Oliveira-Filho RM, Chiauzzi VA, Calmo JC, Charreau EH, and Calandra RS (1982). Effects of prolactin on Prostate Androgen Receptos in Male Rats. *J Andrology* **3:** 281–288.

Barash IA, Cheung CC, Weigle DS, Ren H, Kabigting EB, Kuijper JL, Clifton DK, and Steiner RA (1996). Leptin is a metabolic signal to the reproductive system. *Endocrinology* **137:** 3144–7.

Barraclough CA and Wise PM (1982). The role of catecholamines in the regulation of pituitary luteinizing hormone and follicle-stimulating hormone secretion. *Endocr Rev* **3:** 91–119.

Bartlett JM, Charlton HM, Robinson IC, and Nieschlag E (1990). Pubertal development and testicular function in the male growth hormone-deficient rat. *J Endocrinol* **126:** 193–201.

Becu D and Libertun C (1982). Comparative maturation of the regulation of prolactin and thyrotropin by serotonin and thyrotropin-releasing hormone in male and female rats. *Endocrinology* **110:** 1879–84.

Becu D and Libertun C (1983). Serotoninergic involvement in the cimetidine-induced prolactin release. *Endocrinology* **113:** 1980–4.

Becu de Villalobos D, Vacas MI, Cardinali DP, and Libertun C (1984). A developmental study of adenohypophyseal dopaminergic receptors and of haloperidol-induced prolactin release in rats. *Brain Res* **314:** 167–71.

Becu-Villalobos D, Lacau-Mengido IM, Diaz-Torga GS, and Libertun C (1992). Ontogenic studies of the neural control of adenohypophyseal hormones in the rat. II. Prolactin. *Cell Mol Neurobiol* **12:** 1–19.

Benton L, Shan LX, and Hardy MP (1995). Differentiation of adult Leydig cells. *J Steroid Biochem Mol Biol* **53:** 61–8.

Berelowitz M, Szabo M, Frohman LA, Firestone S, Chu L, and Hintz RL (1981). Somatomedin-C mediates growth hormone negative feedback by effects on both the hypothalamus and the pituitary. *Science* **212:** 1279–81.

Bero LA, Lurie SN, and Kuhn CM (1987). Early ontogeny of kappa-opioid receptor regulation of prolactin secretion in the rat. *Brain Res* **465:** 189–96.

Bhanot R and Wilkinson M (1983). Opiatergic control of gonadotropin secretion during puberty in the rat: a neurochemical basis for the hypothalamic 'gonadostat'? *Endocrinology* **113:** 596–603.

Biegel LB, Cook JC, O'Connor JC, Aschiero M, Arduengo AJ, 3rd, and Slone TW (1995). Subchronic toxicity study in rats with 1-methyl-3-propylimidazole-2- thione (PTI): effects on the thyroid. *Fundam Appl Toxicol* **27:** 185–94.

Biegel LB, Flaws JA, Hirshfield AN, O'Connor JC, Elliott GS, Ladics GS, Silbergeld EK, Van Pelt CS, Hurtt ME, Cook JC, and Frame SR (1998). 90-day feeding and one-generation reproduction study in Crl:CD BR rats with 17 beta-estradiol. *Toxicol Sci* **44:** 116–42.

Bjerke DL and Peterson RE (1994). Reproductive toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in male rats: different effects of in utero vs. lactational exposure. *Toxicol Appl Pharmacol* **127:** 241–9.

Blank MS, Panerai AE, and Friesen HG (1979). Opioid peptides modulate luteinizing hormone secretion during sexual maturation. *Science* **203:** 1129–31.

Blank MS, Panerai AE, and Friesen HG (1980). Effects of naloxone on luteinizing hormone and prolactin in serum of rats. *J Endocrinol* **85:** 307–15.

Bohnet HG, Aragona C, and Friesen HG (1976). Age and sex differences in prolactin response to phenothiazines in rats. *Biol Reprod* **15:** 168–72.

Bourguignon JP, Alvarez Gonzalez ML, Gerard A, and Franchimont P (1994). Gonadotropin releasing hormone inhibitory autofeedback by subproducts antagonist at *N*-methyl-D-aspartate receptors: a model of autocrine regulation of peptide secretion. *Endocrinology* **134:** 1589–92.

Bourguignon JP and Franchimont P (1984). Puberty-related increase in episodic LHRH release from rat hypothalamus in vitro. *Endocrinology* **114:** 1941–3.

Bourguignon JP, Gerard A, Alvarex-Gonzalez ML, Fawe L, and Franchimont P (1992) Gonadal-independent developmental changes in activation of *N*-methyl-D- aspartate receptors involved in gonadotropin-releasing hormone secretion. *Neuroendocrinology* **55:** 634–41.

Bourguignon JP, Gerard A, Mathieu J, Mathieu A, and Franchimont P (1990). Maturation of the hypothalamic control of pulsatile gonadotropin-releasing hormone secretion at onset of puberty. I. Increased activation of *N*-methyl-D-aspartate receptors. *Endocrinology* **127:** 873–81.

Brann DW and Mahesh VB (1995). Glutamate: a major neuroendocrine excitatory signal mediating steroid effects on gonadotropin secretion. *J Steroid Biochem Mol Biol* **53:** 325–9.

Bulger WH, Muccitelli RM, and Kupfer D (1978). Interactions of methoxychlor, methoxychlor base-soluble contaminant, and 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane with rat uterine estrogen receptor. *J Toxicol Environ Health* **4:**881–93.

Buzek SW and Sanborn BM (1988). Increase in testicular androgen receptor during sexual maturation in the rat. *Biol Reprod* **39:** 39–49.

Cagen SZ, Waechter JM, Jr., Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE, and Harris LR (1999). Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A [In Process Citation]. *Toxicol Sci* **50:** 36–44.

Campfield LA, Smith FJ, Mackie G, Tenenbaum R, Sassano ML, Mullin J, Kaiser K, and Kierstead RW (1995). Insulin normalization as an approach to the pharmacological treatment of obesity. *Obes Res* **3 Suppl 4:** 591S–603S.

Capen CC (1997). Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. *Toxicol Pathol* **25:** 39–48.

Capen CC (1998). Correlation of mechanistic data and histopathology in the evaluation of selected toxic endpoints of the endocrine system. *Toxicol Lett* **102-103:** 405–9.

Casanova M, You L, Gaido KW, Archibeque-Engle S, Janszen DB, and Heck HA (1999). Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors alpha and beta in vitro. *Toxicol Sci* **51:** 236–44.

Cavallo A and Dolan LM (1996). 6-Hydroxymelatonin sulfate excretion in human puberty. *J Pineal Res* **21:** 225–30.

Chang CL (1984). The effect of pituitary homografts on the accessory sex organs and serum hormonal levels with or without androgen in mature male rats. *Proc Natl Sci Counc Repub China [B]* **8:** 129–34.

Chapin RE, Davis BJ, Delaney JC, Kaiser LB, Wang Y, Lanning LL, and Wolfe GW (1998). Multigenerational study of 4-nonylphenol in rats. *The Toxicologist* **42:** 100.

Chapin RE, Harris MW, Davis BJ, Ward SM, Wilson RE, Mauney MA, Lockhart AC, Smialowicz RJ, Moser VC, Burka LT, and Collins BJ (1997). The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. *Fundam Appl Toxicol* **40:** 138–57.

Charreau EH, Attramadal A, Torjesen PA, Purvis K, Calandra R, and Hansson V (1977). Prolactin binding in rat testis: specific receptors in interstitial cells. *Mol Cell Endocrinol* **6:** 303–7.

Cheung CC, Clifton DK, and Steiner RA (1997). Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinology* **138:** 4489–92.

Chiappa SA and Fink G (1977). Releasing factor and hormonal changes in the hypothalamic-pituitary-gonadotrophin and -adrenocorticotrophin systems before and after birth and puberty in male, female and androgenized female rats. *J Endocrinol* **72:** 211–24.

Ching M (1981). Effect of barbital on the pituitary-thyroid axis. *J Endocrinol Invest* **4:** 389–92.

Choi S and Kellogg CK (1992). Norepinephrine utilization in the hypothalamus of the male rat during adolescent development. *Dev Neurosci* **14:** 369–76.

Chowdury A, Gautam AK, and Chatterjee BB (1984). Thyroid-testis interrelationship during the development and sexual maturit. *Arch Androl* **13:** 233–239.

Cicero TJ, Adams ML, Giordano A, Miller BT, O'Connor L, and Nock B (1991). Influence of morphine exposure during adolescence on the sexual maturation of male rats and the development of their offspring. *J Pharmacol Exp Ther* **256:** 1086–93.

Cicero TJ, Adams ML, O'Connor L, Nock B, Meyer ER, and Wozniak D (1990). Influence of chronic alcohol administration on representative indices of puberty and sexual maturation in male rats and the development of their progeny. *J Pharmacol Exp Ther* **255:** 707–15.

Cicero TJ, Nock B, and O'Connor L (1993). Naloxone does not reverse the inhibitory effect of morphine on luteinizing hormone secretion in prepubescent male rats. *J Pharmacol Exp Ther* **264:** 47–53.

Cicero TJ, Schmoeker PF, Meyer ER, Miller BT, Bell RD, Cytron SM, and Brown CC (1986). Ontogeny of the opioid-mediated control of reproductive endocrinology in the male and female rat. *J Pharmacol Exp Ther* **236:** 627–33.

Clegg E (1960). The age at which male rats become fertile. *J Reproduct Fertil* **1**.

Clermont V and Perey B (1957). Quantitative study of the cell population of the seminiferous tubules in immature rats. *Am J Anatomy* **100:** 241–268.

Coert A, Nievelstein H, Kloosterboer HJ, Loonen P, and van der Vies J (1985). Effects of hyperprolactinemia on the accessory sexual organs of the male rat. *Prostate* **6:** 269–76.

Collu R, Letarte J, Leboeuf F, and Ducharme JR (1975). Endocrine effects of chronic administration of psychoactive drugs to prepuberal male rats. I. delta9-tetrahydrocannabinol. *Life Sci* **16:** 533–42.

Commentz JC, Uhlig H, Henke A, Hellwege HH, and Willig RP (1997). Melatonin and 6-hydroxymelatonin sulfate excretion is inversely correlated with gonadal development in children. *Horm Res* **47:** 97–101.

Cook JC, Klinefelter GR, Hardisty JF, Sharpe RM, and Foster PM (1999). Rodent Leydig cell tumorigenesis: a review of the physiology, pathology, mechanisms, and relevance to humans. *Crit Rev Toxicol* **29:** 169–261.

Cooke PS and Meisami E (1991). Early hypothyroidism in rats causes increased adult testis and reproductive organ size but does not change testosterone levels. *Endocrinology* **129:** 237–43.

Cooke PS, Porcelli J, and Hess RA (1992). Induction of increased testis growth and sperm production in adult rats by neonatal administration of the goitrogen propylthiouracil (PTU): the critical period. *Biol Reprod* **46:** 146–54.

Cooke PS, Zhao YD, and Hansen LG (1996). Neonatal polychlorinated biphenyl treatment increases adult testis size and sperm production in the rat. *Toxicol Appl Pharmacol* **136:** 112–7.

Copeland KC, DeSouza MM, and Gibson PC (1990). Influence of gonadal steroids on rat pituitary growth hormone secretion. *Res Exp Med* **190:** 137–43.

Corder R, Castagne V, Rivet JM, Mormede P, and Gaillard RC (1992). Central and peripheral effects of repeated stress and high NaCl diet on neuropeptide Y. *Physiol Behav* **52:** 205–10.

Crisp TM, Clegg ED, Cooper RL, Wood WP, Anderson DG, Baetcke KP, Hoffmann JL, Morrow MS, Rodier DJ, Schaeffer JE, Touart LW, Zeeman MG, and Patel YM (1998). Environmental endocrine disruption: an effects assessment and analysis. *Environ Health Perspect* **106 Suppl 1:** 11–56.

Crowley W, Parker. SL, Sahu. A, and Kalra SP (1995). Interacting tansmembrane signals regulating GnRH and LH secretion. In: TM Plant and PA Lee (Eds.): *The Neurobiology of Puberty*, pp. 41–54. Bristol, UK.

Dalkin AC, Bourne GA, Pieper DR, Regiani S, and Marshall JC (1981). Pituitary and gonadal gonadotropin-releasing hormone receptors during sexual maturation in the rat. *Endocrinology* **108:** 1658–64.

Dalla Valle L, Ramina A, Vianello S, Belvedere P, and Colombo L (1996). Kinetic analysis of duodenal and testicular cytochrome P450c17 in the rat. *J Steroid Biochem Mol Biol* **58:** 577–84.

de Jong R and van der Schoot P (1979). Advancement of sexual maturation in male rats by pituitary transplants. *Biol Reproduct* **5:** 1263–71.

de Kretser DM and Kerr JB (1994). The cytology of the testis. In: E Knobil and JD Neill (Eds.): *The Physiology of Reproduction*, 2nd ed. New York: Raven Press, Ltd.

Debeljuk L, Arimura A, and Schally AV (1972). Studies on the pituitary responsiveness to luteinizing hormone-releasing hormone (LH-RH) in intact male rats of different ages. *Endocrinology* **90:** 585–8.

DeVito M, Biegel L, Brouwer A, Brown S, Brucker-Davis F, Cheek AO, Christensen R, Colborn T, Cooke P, Crissman J, Crofton K, Doerge D, Gray E, Hauser P, Hurley P, Kohn M, Lazar J, McMaster S, McClain M, McConnell E, Meier C, Miller R, Tietge J, and Tyl R (1999). Screening methods for thyroid hormone disruptors. *Environ Health Perspect* **107:** 407–415.

Dohler KD and Wuttke W (1974). Serum LH, FSH, prolactin and progesterone from birth to puberty in female and male rats. *Endocrinology* **94:**1003–8.

Dohler KD and Wuttke W (1975). Changes with age in levels of serum gonadotropins, prolactin and gonadal steroids in prepubertal male and female rats. *Endocrinology* **97:** 898–907.

Dullaart J (1977). Immature rat pituitary glands in vitro: age- and sex-related changes in luteinizing hormone releasing hormone-stimulated gonadotrophin release. *J Endocrinol* **73:** 309–19.

Duncan JA, Dalkin AC, Barkan A, Regiani S, and Marshall JC (1983). Gonadal regulation of pituitary gonadotropin-releasing hormone receptors during sexual maturation in the rat. *Endocrinology* **113:** 2238–46.

Dupont E, Labrie F, Luu-The V, and Pelletier G (1993). Ontogeny of 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD) in rat testis as studied by

immunocytochemistry. *Anat Embryol (Berlin)* **187:** 583–9.

Ellis LC (1972). Inhibition of rat testicular androgen synthesis in vitro by melantonin and serotonin. *Endocrinology* **90:** 17–28.

Emanuele MA, LaPaglia N, Steiner J, Jabamoni K, Hansen M, Kirsteins L, and Emanuele NV (1998). Reversal of ethanol-induced testosterone suppression in peripubertal male rats by opiate blockade. *Alcohol Clin Exp Res* **22:** 1199–204.

Evans NP and Karsch FJ (1995). Operation of the gonadotropin-releasing hormone pulse generator. In: *The Neurobiology of Puberty*. Great Britain: Bourne Press.

Feleder C, Jarry H, Leonhardt S, Wuttke W, and Moguilevsky JA (1996). The GABAergic control of gonadotropin-releasing hormone secretion in male rats during sexual maturation involves effects on hypothalamic excitatory and inhibitory amino acid systems. *Neuroendocrinology* **64:** 305–12.

Foster PM, Thomas LV, Cook MW, and Walters DG (1983). Effect of DI-*n*-pentyl phthalate treatment on testicular steroidogenic enzymes and cytochrome P-450 in the rat. *Toxicol Lett* **15:** 265–71.

Francavilla S, Cordeschi G, Properzi G, Di Cicco L, Jannini EA, Palmero S, Fugassa E, Loras B, and D'Armiento M (1991). Effect of thyroid hormone on the pre- and postnatal development of the rat testis. *J Endocrinol* **129:** 35–42.

Franklin RB, Zou J, Gorski E, Yang YH, and Costello LC (1997). Prolactin regulation of mitochondrial aspartate aminotransferase and protein kinase C in human prostate cancer cells. *Mol Cell Endocrinol* **127:** 19–25.

Freitag F and Docke F (1987). Differential effects of chronic testosterone treatment on the onset of puberty in male rats. *Exp Clin Endocrinol* **90:** 361–364.

Fukuda H and Greer MA (1978). Postnatal development of pituitary-thyroid function in male and female rats: comparison of plasma and thyroid T3 and T4 concentration. *J Endocrinol Invest* **1:** 311–4.

Gabriel SM, Roncancio JR, and Ruiz NS (1992). Growth hormone pulsatility and the endocrine milieu during sexual maturation in male and female rats. *Neuroendocrinology* **56:** 619–25.

Gayo L, Bonet B, Herranz AS, Iglesias R, Toro MJ, and Montoya E (1986). Postnatal development of brain TRH, serum TSH and thyroid hormones in the male and female rat. *Acta Endocrinol (Copenhagen)* **112:** 7–11.

Gaytan F, Bellido C, Aguilar R, and Aguilar E (1988). Balano-preputial separation as an external sign of puberty in the rat: correlation with histologic testicular data. *Andrologia* **20:** 450–3.

Ge RS and Hardy MP (1998). Variation in the end products of androgen biosynthesis and matabolism during postnatal differentiation of rat Leydig cells. *Endocrinology* **139:** 3787–95.

George FW and Wilson JD (1994). Sex determination and differentiation. In: E Knobil and JD Neill (Eds.): *The Physiology of Reproduction*, 2nd Ed. New York: Raven Press, Ltd.

Ghosh PK, Biswas NM, and Ghosh D (1991). Effect of lithium chloride on testicular steroidogenesis and gametogenesis in immature male rats. *Acta Endocrinol (Copenhagen)* **124:** 76–82.

Glass AR, Anderson J, and Herbert D (1987). Sexual maturation in underfed weight-matched rats. A test of the "critical body weight" theory of pubertal timing in males. *J Androl* **8:** 116–22.

Goldman AS and Klingele DA (1974). Developmental defects of testicular morphology and steroidogenesis in the male rat pseudohermaphrodite and response to testosterone and dihydrotestosterone. *Endocrinology* **94:** 1–16.

Goldsmith PC, Thind KK, Perera AD, and Plant TM (1994). Glutamate-immunoreactive neurons and their gonadotropin-releasing hormone-neuronal interactions in the monkey hypothalamus. *Endocrinology* **134:** 858–68.

Gray LE, Jr. and Kelce WR (1996). Latent effects of pesticides and toxic substances on sexual differentiation of rodents. *Toxicol Ind Health* **12:** 515–31.

Gray LE, Jr., Kelce WR, Monosson E, Ostby JS, and Birnbaum LS (1995). Exposure to TCDD during development permanently alters reproductive function in male Long Evans rats and hamsters: reduced ejaculated and epididymal sperm numbers and sex accessory gland weights in offspring with normal androgenic status. *Toxicol Appl Pharmacol* **131:** 108–18.

Gray LE, Jr and Ostby J (1998). Effects of pesticides and toxic substances on behavioral and morphological reproductive development: endocrine versus nonendocrine mechanisms. *Toxicol Ind Health* **14:** 159–84.

Gray LE and Ostby J (1999). Profilds of effects of antiandrogenic pesticides and toxic substances on sex differentiation. *The Toxicologist* **48**.

Gray LE, Jr, Ostby J, Ferrell J, Rehnberg G, Linder R, Cooper R, Goldman J, Slott V, and Laskey J (1989). A dose-response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundam Appl Toxicol* **12:** 92–108.

Gray LE, Jr, Ostby JS, Ferrell JM, Sigmon ER, and Goldman JM (1988). Methoxychlor induces estrogen-like alterations of behavior and the reproductive tract in the female rat and hamster: effects on sex behavior, running wheel activity, and uterine morphology. *Toxicol Appl Pharmacol* **96:** 525–40.

Gray LE, Jr., Wolf C, Lambright C, Mann P, Price M, Cooper RL, and Ostby J (1999). Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, *p,p′*-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health* **15:** 94–118.

Gray TJ, Rowland IR, Foster PM, and Gangolli SD (1982). Species differences in the testicular toxicity of phthalate esters. *Toxicol Lett* **11:** 141–7.

Grizard G (1994). [IGF(s) and testicular functions. Secretion and action of IGF-1 on Leydig cells]. *Contracept Fertil Sex* **22:** 551–5.

Gruaz NM, Pierroz DD, Rohner-Jeanrenaud F, Sizonenko PC, and Aubert ML (1993). Evidence that neuropeptide Y could represent a neuroendocrine inhibitor of sexual

maturation in unfavorable metabolic conditions in the rat. *Endocrinology* **133:** 1891–4.

Gupta D and Elbracht C (1983). Effect of tetrahydrocannabinols on pubertal body weight spurt and sex hormones in developing male rats. *Res Exp Med* **182:** 95–104.

Gupta D, Zarzycki J, and Rager K (1975). Plasma testosterone and dihydrotestosterone in male rats during sexual maturation and following orchidectomy and experimental bilateral cryptorchidism. *Steroids* **25:** 33–42.

Guzelian PS (1992). The clinical toxicology of chlordecone as an example of toxicological risk assessment for man. *Toxicol Lett* **64-65 Spec No:** 589–96.

Hadley ME (1996). *Endocrinology*. Upper Saddle River, NJ: Prentice-Hall, Inc.

Hafiez AA, Philpott JE, and Bartke A (1971). The role of prolactin in the regulation of testicular function: the effect of prolactin and luteinizing hormone on 3-hydroxysteroid dehydrogenase activity in the testes of mice and rats. *J Endocrinol* **50:** 619–23.

Hall PF (1994). Testicular steroid synthesis: organization and regulation. In: E Knobil and JD Neill (Eds.): *The Physiology of Reproduction*, 2nd ed. New York: Raven Press, Ltd.

Handelsman DJ, Spaliviero JA, Scott CD, and Baxter RC (1987). Hormonal regulation of the peripubertal surge of insulin-like growth factor-I in the rat. *Endocrinology* **120:** 491–6.

Hardy MP, Gelber SJ, Zhou ZF, Penning TM, Ricigliano JW, Ganjam VK, Nonneman D, and Ewing LL (1991). Hormonal control of Leydig cell differentiation. *Ann NY Acad Sci* **637:** 152–63.

Hardy MP, Kelce WR, Klinefelter GR, and Ewing LL (1990). Differentiation of Leydig cell precursors in vitro: a role for androgen. *Endocrinology* **127:** 488–90.

Hardy MP, Sharma RS, Arambepola NK, Sottas CM, Russell LD, Bunick D, Hess RA, and Cooke PS (1996). Increased proliferation of Leydig cells induced by neonatal hypothyroidism in the rat. *J Androl* **17:** 231–8.

Hardy MP, Zirkin BR, and Ewing LL (1989). Kinetic studies on the development of the adult population of Leydig cells in testes of the pubertal rat. *Endocrinology* **124:** 762–70.

Hedner T and Lundborg P (1981). Neurochemical characteristics of cerebral catecholamine neurons during the postnatal development in the rat. *Med Biol* **59:** 212–23.

Hiney JK, Ojeda SR, and Dees WL (1991). Insulin-like growth factor I: a possible metabolic signal involved in the regulation of female puberty. *Neuroendocrinology* **54:** 420–3.

Holland JM and Lee C (1980). Effects of pituitary grafts on testosterone stimulated growth of rat prostate. *Biol Reprod* **22:** 351–5.

Hostetter MW and Piacsek BE (1977). The effect of prolactin deficiency during sexual maturation in the male rat. *Biol Reprod* **17:** 574–7.

Hou Y and Voogt JL (1999). Effects of naloxone infusion on nocturnal prolactin secretion and Fos/FRA expression in pregnant rats. *Endocrine* **10:** 145–52.

Huhtaniemi IT, Nevo N, Amsterdam A, and Naor Z (1986). Effect of postnatal treatment with a gonadotropin-releasing hormone antagonist on sexual maturation of male rats. *Biol Reprod* **35:** 501–7.

Huhtaniemi IT, Nozu K, Warren DW, Dufau ML, and Catt KJ (1982). Acquisition of regulatory mechanisms for gonadotropin receptors and steroidogenesis in the maturing rat testis. *Endocrinology* **111:** 1711–20.

Huhtaniemi IT, Stewart JM, Channabasavaiah K, Fraser HM, and Clayton RN (1984). Pituitary-testicular function in immature rats after treatment with GnRH antagonist, GnRH antiserum and bromocriptine. *Mol Cell Endocrinol* **34:** 137–43.

Jannini EA, Ulisse S, Piersanti D, Carosa E, Muzi P, Lazar J, and D'Armiento M (1993). Early thyroid hormone treatment in rats increases testis size and germ cell number. *Endocrinology* **132:** 2726–8.

Jennes L, Stumpf WE, and Sheedy ME (1985). Ultrastructural characterization of gonadotropin-releasing hormone (GnRH)-producing neurons. *J Comp Neurol* **232:** 534–47.

Johnson DC and Brown EB, Jr. (1974). Temporal augmentation of LH by prolactin in stimulation of androgen production by the testes of hypophysectomized male rats. *Proc Soc Exp Biol Med* **145:** 610–3.

Johnston CA and Negro-Vilar A (1986). Maturation of the prolactin and proopiomelanocortin-derived peptide responses to ether stress and morphine: neurochemical analysis. *Endocrinology* **118:** 797–804.

Johnston LD, O'Malley PM, and Bachman JG (1987). Psychotherapeutic, licit, and illicit use of drugs among adolescents. An epidemiological perspective. *J Adolesc Health Care* **8:** 36–51.

Jordan D, Rousset B, Perrin F, Fournier M, and Orgiazzi J (1980). Evidence for circadian variations in serum thyrotropin, 3,5,3′- triiodothyronine, and thyroxine in the rat. *Endocrinology* **107:** 1245–8.

Jordan D, Veisseire M, Borson-Chazot F, and Mornex R (1987). Postnatal development of TRH and TSH rhythms in the rat. *Horm Res* **27:** 216–24.

Kamberi IA, de Vellis J, Bacleon ES, and Inglish D (1980). Hormonal patterns of the hypothalamo-pituitary-gonadal axis in the rat during postnatal development and sexual maturation. *Endokrinologie* **75:** 129–40.

Kapil N, Chowdhury AR, and Swarup H (1979). Role of ACTH on the cytomorphology of testes of the immature rat. *Experientia* **35:** 1606–7.

Karanth S, Dutt A, and Juneja HS (1987). Age-related release of prolactin by the pituitary and the pituitary- hypothalamic complex in vitro: an attempt to describe the development of the hypothalamic prolactin-inhibiting and -releasing activities in male rats. *J Endocrinol* **115:** 405–9.

Keenan EJ and Thomas JA (1975). Effects of testosterone and prolactin or growth hormone on the accessory sex organs of castrated mice. *J Endocrinol* **64:** 111–5.

Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, and Wilson EM (1995). Persistent DDT metabolite *p,p′*-DDE is a potent androgen receptor antagonist. *Nature* **375:** 581–5.

Kennedy GC and Mitra J (1963). Body weight and food intake as initiating factors for puberty in the rat. *Physiology* **166:** 408–418.

Ketelslegers JM, Hetzel WD, Sherins RJ, and Catt KJ (1978). Developmental changes in testicular gonadotropin receptors: plasma gonadotropins and plasma testosterone in the rat. *Endocrinology* **103:** 212–22.

Kimura F and Kawakami M (1982). Episodic lh secretion in the immature male and female rat as assessed by sequential blood sampling. *Neuroendocrinology* **35:** 128–32.

Kolbusz WE, Lee C, and Grayhack JT (1982). Delay of castration induced regression in rat prostate by pituitary homografts. *J Urol* **127:** 581–4.

Kolena J and Sebokova E (1983). Hormonal regulation of testicular LH/hCG receptors in rat. *Exp Clin Endocrinol* **82:** 1–7.

Kolho KL, Nikula H, and Huhtaniemi I (1988). Sexual maturation of male rats treated postnatally with a gonadotrophin-releasing hormone antagonist. *J Endocrinol* **116:** 241–6.

Korenbrot CC, Huhtaniemi IT, and Weiner RI (1977). Preputial separation as an external sign of pubertal development in the male rat. *Biol Reprod* **17:** 298–303.

Kovacevic R, Krsmanovic L, Stojilkovic S, Simonovic I, Maric D, and Andjus RK (1982). Effects of bromocriptine-induced hypoprolactinaemia on the developmental pattern of androgen and LH levels in the male rat. *Int J Androl* **5:** 437–47.

Krall A and Spring-Mills E (1982). Effects of perphenazine and bromocriptine on male accessory sex glands. III. The prostate gland. *Arch Androl* **9:** 273–8.

Kuhn CM and Schanberg SM (1981). Maturation of central nervous system control of growth hormone secretion in rats. *J Pharmacol Exp Ther* **217:** 152–6.

Lacau de Mengido IM, Diaz-Torga GS, and Libertun C (1989). Diazepam: endocrine effects and hypothalamic binding sites in the developing male and female rat. *Life Sci* **45:** 567–75.

Lambright CR, Hotchkiss AK, and Gray ELJ (1999). Cellular and molecular mechanisms of action of three novel environmental antiandrogens: two phthalates and linuron. *The Toxicologist* **48**.

Lamprecht F and Coyle JT (1972). DOPA decarboxylase in the developing rat brain. *Brain Res* **41:** 503–6.

Lang U, Aubert ML, Conne BS, Bradtke JC, and Sizonenko PC (1983). Influence of exogenous melatonin on melatonin secretion and the neuroendocrine reproductive axis of intact male rats during sexual maturation. *Endocrinology* **112:** 1578–84.

Lang U, Aubert ML, Rivest RW, Vinas-Bradtke JC, and Sizonenko PC (1985). Inhibitory action of exogenous melatonin, 5-methoxytryptamine, and 6-hydroxymelatonin on sexual maturation of male rats: activity of 5-methoxytryptamine might be due to its conversion to melatonin. *Biol Reprod* **33:** 618–28.

Lang U, Rivest RW, Schlaepfer LV, Bradtke JC, Aubert ML, and Sizonenko PC (1984). Diurnal rhythm of melatonin action on sexual maturation of male rats. *Neuroendocrinology* **38:** 261–8.

Laudon M, Hyde JF, and Ben-Jonathan N (1989). Ontogeny of prolactin releasing and inhibiting activities in the posterior pituitary of male rats. *Neuroendocrinology* **50:** 644–9.

Lee VWK, de Kretser DM, Hudson B, and Wang C (1975). Variations in serum FSH, LH and testosterone levels in male rats from birth to sexual maturity. *J Reprod Fertil* **42:** 121–6.

Lephart ED and Ojeda SR (1990). Hypothalamic aromatase activity in male and female rats during juvenile peripubertal development. *Neuroendocrinology* **51:** 385–93.

Lin T, Blaisdell J, and Haskell JF (1987). Type I IGF receptors of Leydig cells are upregulated by human chorionic gonadotropin. *Biochem Biophys Res Commun* **149:** 852–8.

Lin T, Blaisdell J, and Haskell JF (1988). Hormonal regulation of type I insulin-like growth factor receptors of Leydig cells in hypophysectomized rats. *Endocrinology* **123:** 134–9.

Luke MC and Coffey DS (1994). The male asseccory tissues. In: E Knobil and JD Neill (Eds.): *The Physiology of Reproduction*, 2nd ed. New York: Raven Press, Ltd.

Lyons WR, Berlin I, and Friendlander S (1942). Cornification of balano-preputial epithelius in normal rats and in castrated rats treated with testosterone proprionate. *Endocrinology* **31:** 659.

Macfarland LA and Mann DR (1977). The inhibitory effects of ACTH and adrenalectomy on reproductive maturation in female rats. *Biol Reprod* **16:** 306–14.

Maric D, Stojilkovic S, Krsmanovic L, Simonovic I, Kovacevic R, and Andjus RK (1987). Rapid naloxone-induced alterations of androgen variables in the growing male rat. *Neuroendocrinology* **46:** 167–75.

Marsh JM (1976). The role of cyclic AMP in gonadal steroidogenesis. *Biol Reprod* **14:** 30–53.

Marshall (1966). *Marshall's Physiology of Reproduction*. Longmans, Green and Co. LTD.

Martin JE and Sattler C (1982). Selectivity of melatonin pituitary inhibition for luteinizing hormone-releasing hormone. *Neuroendocrinology* **34:** 112–6.

Martini L, Celotti F, Massa R, and Motta M (1978). Studies on the mode of action of androgens in the neuroendocrine tissues. *J Steroid Biochem* **9:** 411–7.

Matsumoto A and Arai Y (1981). Effect of androgen on sexual differentiation of synaptic organization in the hypothalamic arcuate nucleus: an ontogenetic study. *Neuroendocrinology* **33:** 166–9.

Matsumoto AM, Karpas AE, Southworth MB, Dorsa DM, and Bremner WJ (1986). Evidence for activation of the central nervous system-pituitary mechanism for gonadotropin secretion at the time of puberty in the male rat. *Endocrinology* **119:** 362–9.

McCann SM, Ojeda SR, Fawcett CP, and Krulich L (1974). Catecholaminergic control of gonadotropin and prolactin secretion with particular reference to the possible participation of dopamine. *Adv Neurol* **5:** 435–45.

McClain RM, Posch RC, Bosakowski T, and Armstrong JM (1988). Studies on the mode of action for thyroid gland tumor promotion in rats by phenobarbital. *Toxicol Appl Pharmacol* **94:** 254–65.

McNeilly AS, de Kretser DM, and Sharpe RM (1979). Modulation of prolactin, luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion by LHRH and bromocriptine (CB154) in the hypophysectomized pituitary-grafted male rat and its effect on testicular LH receptors and testosterone output. *Biol Reprod* **21:** 141–7.

Meisami E, Sendera TJ, and Clay LB (1992). Paradoxical hypertrophy and plasticity of the testis in rats recovering from early thyroid deficiency: a growth study including effects of age and duration of hypothyroidism. *J Endocrinol* **135:** 495–505.

Moger WH (1977). Serum 5alpha-androstane-3alpha,17beta-diol, androsterone, and testosterone concentrations in the male rat. Influence of age and gonadotropin stimulation. *Endocrinology* **100:** 1027–32.

Monosson E, Kelce WR, Lambright C, Ostby J, and Gray LE, Jr. (1999). Peripubertal exposure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat [In Process Citation]. *Toxicol Ind Health* **15:** 65–79.

Mosotto C and Negro-Vilar A (1987). Activation of gamma-amino butyric acid B-receptors abolishes naloxone-stimulated luteinizing hormone release. *Endocrinology* **121:** 2251–5.

Murono EP and Payne AH (1979). Testicular maturation in the rat. In vivo effect of gonadotropins on steroidogenic enzymes in the hypophysectomized immature rat. *Biol Reprod* **20:** 911–7.

Mylchreest E and Foster PMD (1998). *Antiandrogenic Effects of Di(n-Butyl) Phthalate on Male Reproductive Development: A Nonreceptor-Mediated Mechanism*. Chemical Industry Institute of Toxicology Activities, Sept. Vol. 18, No. 9.

Naftolin F, Morishita H, Davies IJ, Todd R, and Ryan KJ (1975). 2-hydroxyestrone induced rise in serum luteinizing hormone in the immature male rat. *Biochem Biophys Res Commun* **64:** 905–10.

Nagpal ML, Wang D, Calkins JH, Chang WW, and Lin T (1991). Human chorionic gonadotropin up-regulates insulin-like growth factor-I receptor gene expression of Leydig cells. *Endocrinology* **129:** 2820–6.

Naumenko EV and Serova LI (1976) Diencephalic dopamine receptors and regulation of pituitary-testicular system. *Brain Res* **110:** 537–45.

Nazian SJ (1983). Pituitary function during the sexual maturation of the male rat: inhibition of prolactin secretion by exogenous dopamine. *Biol Reprod* **28:** 645–51.

Nazian SJ (1992). Development and control of the opioid inhibition of gonadotropin secretion during the sexual maturation of the male rat. *Neuroendocrinology* **55:** 613–20.

Nazian SJ and Mahesh VB (1979). Sexual maturation of the male rat. Influence of androgens on the pituitary response to single or multiple injections of LH-RH. *Neuroendocrinology* **29:** 313–22.

Nazian SJ and Mahesh VB (1980). Hypothalamic, pituitary, testicular, and secondary organ functions and interactions during the sexual maturation of the male rat. *Arch Androl* **4:** 283–303.

Negro-Vilar A, Krulich L, and McCann SM (1973a). Changes in serum prolactin and gonadotropins during sexual development of the male rat. Endocrinology *93:*660-4.

Negro-Vilar A, Ojeda SR, and McCann SM (1973b) Evidence for changes in sensitivity to testosterone negative feedback on gonadotropin release during sexual development in the male rat. *Endocrinology* **93:** 729–35.

Negro-Vilar A, Saad WA, and McCann SM (1977). Evidence for a role of prolactin in prostate and seminal vesicle growth in immature male rats. *Endocrinology* **100:** 729–37.

O'Connor JC, Cook JC, Slone TW, Makovec GT, Frame SR, and Davis LG (1998). An ongoing validation of a Tier I screening battery for detecting endocrine-active compounds (EACs). *Toxicol Sci* **46:** 45–60.

Odell WD and Swerdloff RS (1976). Etiologies of sexual maturation: a model system based on the sexually maturing rat. *Recent Prog Horm Res* **32:** 245–88.

Odell WD, Swerdloff RS, Jacobs HS, and Hescox MA (1973). FSH induction of sensitivity to LH: one cause of sexual maturation in the male rat. *Endocrinology* **92:** 160–5.

Oishi S (1985). Reversibility of testicular atrophy induced by Di(2-ethylhexyl) phthalate in rats. *Environ Res* **36:** 160–9.

Ojeda SR, Andrews WW, Advis JP, and White SS (1980). Recent advances in the endocrinology of puberty. *Endocr Rev* **1:** 228–57.

Ojeda SR and Ma YJ (1998). Epidermal growth factor tyrosine kinase receptors and the neuroendocrine control of mammalian puberty. *Mol Cell Endocrinol* **140:** 101–6.

Ojeda SR and Ramirez VD (1972). Plasma level of LH and FSH in maturing rats: response to hemigonadectomy. *Endocrinology* **90:** 466–72.

Ojeda SR and Urbanski HF (1994). Puberty in the rat. In: E Knobil and JD Neill (Eds.): *The Physiology of Reproduction*, 2nd ed. New York: Raven Press.

Olea N, Pulgar R, Perez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, and Soto A (1996). Estrogenicity of resin-based composites and sealants used in dentistry. *Environ Health Perspect* **104:** 298–305.

Pallardo LF, Pericas I, and Jolin T (1976). Thyroid iodine uptake, thyroid iodine secretion and plasma TSH levels in male rats during the day and night. *Acta Endocrinol (Copenhagen)* **82:** 517–29.

Palmero S, Prati M, De Marco P, Trucchi P, and Fugassa E (1993). Thyroidal regulation of nuclear tri-iodothyronine receptors in the developing rat testis. *J Endocrinol* **136:** 277–82.

Palmero S, Trucchi P, Prati M, Fugassa E, Lanni A, and Goglia F (1994). Effect of thyroid status on the oxidative capacity of Sertoli cells isolated from immature rat testis. *Eur J Endocrinol* **130:** 308–12.

Payne AH, Kelch RP, Murono EP, and Kerlan JT (1977). Hypothalamic, pituitary and testicular function during sexual maturation of the male rat. *J Endocrinol* **72:** 17–26.

Pazos F, F Sn-F, Balsa J, J Lp-F, Escalada J, and Cacicedo L (1999). Regulation of gonadal and somatotropic axis by chronic intraventricular infusion of insulin-like growth factor 1 antibody at the initiation of puberty in male rats. *Neuroendocrinology 69:*408–416.

Phillip M, Palese T, Hernandez ER, Roberts CT, Jr, LeRoith D, and Kowarski AA (1992). Effect of testosterone on insulin-like growth factor-I (IGF-I) and IGF- I receptor gene expression in the hypophysectomized rat. *Endocrinology* **130:** 2865–70.

Piacsek BE and Goodspeed MP (1978). Maturation of the pituitary-gonadal system in the male rat. *J Reprod Fertil* **52:** 29–35.

Pinilla L, Tena-Sempere M, and Aguilar E (1998). Sexual differences in the role of kainate receptors in controlling gonadotrophin secretion in prepubertal rats. *J Reprod Fertil* **113:** 269–73.

Plant T, Gay V, Marshall G, and Arslan M (1989). Puberty in monkeys in triggered by chemical stimulation of the

hypothalamus. *Proc Natl Acad Sci U.S.A.* **7:** 2506–2510.

Potterger LH, Domoradzki JY, Markham DA, Hansen SC, and Waechter JM, Jr (1998). Metabolites of bisphenol A (BPA) in rat plasma are dependent upon the route of administration. *Toxicol Sci* **42:** 282.

Purvis K, Clausen OP, Olsen A, Haug E, and Hansson V (1979). Prolactin and Leydig cell responsiveness to LH/hCG in the rat. *Arch Androl* **3:** 219–30.

Ramaley JA (1981). Serum prolactin levels in the prepubertal period in male and female rats. Control by photoperiod and gonadal status and relationship to puberty onset. *Int J Androl* **4:** 91–104.

Ramaley JA and Phares CK (1983). Delay of puberty onset in males due to suppression of growth hormone. *Neuroendocrinology* **36:** 321–9.

Ramirez V, Feder HH, and Sawyer CH (1984). *The Role of Brain Catecholamines in the Regulation of LH Secretion: A Critical Inquiry*. New York: Raven Press.

Raum WJ and Swerdloff RS (1986). The effect of hypothalamic catecholamine synthesis inhibition by alpha-methyltyrosine on gonadotropin secretion during sexual maturation in the male Wistar rat. *Endocrinology* **119:** 168–75.

Reiter E, Hennuy B, Bruyninx M, Cornet A, Klug M, McNamara M, Closset J, and Hennen G (1999). Effects of pituitary hormones on the prostate. *Prostate* **38:** 159–65.

Reiter E, Lardinois S, Klug M, Sente B, Hennuy B, Bruyninx M, Closset J, and Hennen G (1995). Androgen-independent effects of prolactin on the different lobes of the immature rat prostate. *Mol Cell Endocrinol* **112:** 113–22.

Reiter RJ (1980). Photoperiod: its importance as an impeller of pineal and seasonal reproductive rhythms. *Int J Biometeorol* **24:**57–63.

Reuber MD (1979). The carcinogenicity kepone. *J Environ Pathol Toxicol* **2:** 671–86.

Reynolds RW and Bryson G (1974). Effect of estradiol on the hypothalamic regulation of body weight in the rat. *Res Commun Chem Pathol Pharmacol* **7:** 715–24.

Ronis MJ, Badger TM, Shema SJ, Roberson PK, and Shaikh F (1996). Reproductive toxicity and growth effects in rats exposed to lead at different periods during development. *Toxicol Appl Pharmacol* **136:**361–71.

Sahu A (1998). Evidence suggesting that galanin (GAL), melanin-concentrating hormone (MCH), neurotensin (NT), proopiomelanocortin (POMC) and neuropeptide Y (NPY) are targets of leptin signaling in the hypothalamus. *Endocrinology* **139:** 795–8.

Saito S (1995). Inhibin functions in male rats. *Arch Androl* **34:** 101–4.

Schafer TE, Lapp CA, Hanes CM, Lewis JB, Wataha JC, and Schuster GS (1999). Estrogenicity of bisphenol A and bisphenol A dimethacrylate in vitro. *J Biomed Mater Res* **45:** 192–197.

Selgas L, Arce A, Esquifino AI, and Cardinali DP (1997). Twenty-four-hour rhythms of serum ACTH, prolactin, growth hormone, and thyroid-stimulating hormone, and of median-eminence norepinephrine, dopamine, and serotonin, in rats injected with Freund's adjuvant. *Chronobiol Int* **14:** 253–65.

Shan LX, Bardin CW, and Hardy MP (1997). Immunohistochemical analysis of androgen effects on androgen receptor expression in developing Leydig and Sertoli cells. *Endocrinology* **138:** 1259–66.

Shan LX, and Hardy MP (1992). Developmental changes in levels of luteinizing hormone receptor and androgen receptor in rat Leydig cells. *Endocrinology* **131:** 1107–14.

Sharpe RM, Turner KJ, McKinnell C, Groome NP, Atanassova N, Millar MR, Buchanan DL, and Cooke PS (1999). Inhibin B levels in plasma of the male rat from birth to adulthood: effect of experimental manipulation of Sertoli cell number [In Process Citation]. *J Androl* **20:** 94–101.

Sheth AR, Vanage GR, Vaze AY, and Thakur AN (1980). Negative feedback of secretion of follicle-stimulating hormone by the pituitary gland of developing male rats. *J Endocrinol* **87:** 401–7.

Simpkins JW, Bruni JF, Mioduszewski RJ, and Meites J (1976). Serum and pituitary TSH and response to TRH in developing male and female rats. *Endocrinology* **98:** 1365–9.

Skinner MK, Norton JN, Mullaney BP, Rosselli M, Whaley PD, and Anthony CT (1991). Cell-cell interactions and the regulation of testis function. *Ann NY Acad Sci* **637:** 354–63.

Sokol RZ and Berman N (1991). The effect of age of exposure on lead-induced testicular toxicity. *Toxicology* **69:** 269–78.

Soldani R, Cagnacci A, Paoletti AM, Yen SS, and Melis GB (1995). Modulation of anterior pituitary luteinizing hormone response to gonadotropin-releasing hormone by insulin-like growth factor I in vitro. *Fertil Steril* **64:** 634–7.

Sonnenschein C and Soto A (1998) *An Updated Review of Environmental Estrogen and Androgen Mimics and Antagonists*. Boston, MA: Tufts University School of Medicine, Department of Anatomy and Cellular Biology.

Srivastava S and Srivastava SP (1991). Effect of di(2-ethylhexyl)phthalate on 17 beta-hydroxysteroid dehydrogenase activity in testis of rat. *Toxicol Lett* **57:** 235–39.

Steinberger E (1976). Biological action of gonadotropins in the male. *Pharmacol Ther [B]* **2:** 771–86.

Steiner JC, LaPaglia N, Hansen M, Emanuele NV, and Emanuele MA (1997). Effect of chronic ethanol on reproductive and growth hormones in the peripubertal male rat. *J Endocrinol* **154:** 363–70.

Stojilkovic SS, Dufau ML, and Catt KJ (1987). Opiate receptor subtypes in the rat hypothalamus and neurointermediate lobe. *Endocrinology* **121:** 384–94.

Stoker TE, Cooper RL, Laws SC, and Robinette CL (1999). Elevated prolactin (PRL) prior to puberty and the development of prostatitis in adult Wistar rats. *The Toxicologist* **48**.

Suescun MO, Gonzalez SI, Chiauzzi VA, and Calandra RS (1985). Effects of induced hypoprolactinemia on testicular function during gonadal maturation in the rat. *J Androl* **6:** 77–82.

Sullivan JN and Strott CA (1973). Evidence for an androgen-independent mechanism regulating the levels of receptor in target tissue. *J Biol Chem* **248:** 3202–8.

Swerdloff RS, Peterson M, Vera A, Batt RA, Heber D, and Bray GA (1978). The hypothalamic-pituitary axis in genetically obese (ob/ob) mice: response to luteinizing hormone-releasing hormone. *Endocrinology* **103:** 542–7.

Swerdloff RS and Walsh PC (1973). Testosterone and oestradiol suppression of LH and FSH in adult male rats: duration of castration, duration of treatment and combined treatment. *Acta Endocrinol (Copenhagen)* **73:** 11–21.

Tentler JJ, LaPaglia N, Steiner J, Williams D, Castelli M, Kelley MR, Emanuele NV, and Emanuele MA (1997). Ethanol, growth hormone and testosterone in peripubertal rats. *J Endocrinol* **152:** 477–87.

Terasawa. E (1995). Mechanisms controlling the onset of puberty in primates: the role of GABAergic neurons. *J. Endocrinol*.

Thigpen JE, Setchell KD, Ahlmark KB, Locklear J, Spahr T, Caviness GF, Goelz MF, Haseman JK, Newbold RR, and Forsythe DB (1999). Phytoestrogen content of purified, open- and closed-formula laboratory animal diets. *Lab Anim Sci* **49:** 530–6.

Tyl RW, Myers CB, Marr MC, Brine DR, Fail PA, Seely JC, and Van Miller JP (1999). Two-generation reproduction study with para-tert-octylphenol in rats. *Regul Toxicol Pharmacol* **30:** 81–95.

Ulisse S, Rucci N, Piersanti D, Carosa E, Graziano FM, Pavan A, Ceddia P, Arizzi M, Muzi P, Cironi L, Gnessi L, D'Armiento M, and Jannini EA (1998). Regulation by thyroid hormone of the expression of basement membrane components in rat prepubertal Sertoli cells. *Endocrinology* **139:** 741–7.

Valle LB, Oliveira-Filho RM, Romaldini JH, and Lara PF (1985). Pituitary-testicular axis abnormalities in immature male hypothyroid rats. *J Steroid Biochem* **23:** 253–7.

van den Dungen HM, Dijkstra H, Hiehle MA, van Rees GP, and Schoemaker J (1989). Effects of LHRH antagonist administration to immature male rats on sexual development. *Physiol Behav* **46:** 779–85.

Van Haaster L, De Jong FH, Docter R, and De Rooij DG (1992). The effect of hypothyroidism on Sertoli cell proliferation and differentiation and hormone levels during testicular development in the rat. *Endocrinology* **131:** 1574–1576.

Vianello S, Waterman MR, Dalla Valle L, and Colombo L (1997). Developmentally regulated expression and activity of 17alpha- hydroxylase/C-17,20-lyase cytochrome P450 in rat liver. *Endocrinology* **138:** 3166–74.

Vihko KK, LaPolt PS, Nishimori K, and Hsueh AJ (1991). Stimulatory effects of recombinant follicle-stimulating hormone on Leydig cell function and spermatogenesis in immature hypophysectomized rats. *Endocrinology* **129:** 1926–32.

Wade GN (1972). Gonadal hormones and behavioral regulation of body weight. *Physiol Behav* **8:** 523–34.

Waites GM, Speight AC, and Jenkins N (1985). The functional maturation of the Sertoli cell and Leydig cell in the mammalian testis. *J Reprod Fertil* **75:** 317–26.

Walker P, Dussault JH, Alvarado-Urbina G, and Dupont A (1977). The development of the hypothalamo-pituitary axis in the neonatal rat: hypothalamic somatostatin and pituitary and serum growth hormone concentrations. *Endocrinology* **101:** 782–7.

Warren DW, Huhtaniemi IT, Tapanainen J, Dufau ML, and Catt KJ (1984). Ontogeny of gonadotropin receptors in the fetal and neonatal rat testis. *Endocrinology* **114:** 470–6.

Wehrenberg WB and Giustina A (1992). Basic counterpoint: mechanisms and pathways of gonadal steroid modulation of growth hormone secretion. *Endocr Rev* **13:**299–308.

Wehrenberg WB, Ling N, Bohlen P, Esch F, Brazeau P, and Guillemin R (1982). Physiological roles of somatocrinin and somatostatin in the regulation of growth hormone secretion. *Biochem Biophys Res Commun* **109:** 562–7.

Wigodsky HS, and Green RR (1940). The effect of testosterone, estrogene, and estradiol applied locally to the penis of the rat. *Endocrinology* **26:** 1078.

Wilen R and Naftolin F (1978). Pubertal food intake and body length, weight, and composition in the feed-restricted female rat: comparison with well fed animals. *Pediatr Res* **12:** 263–7.

Wisner JR, Jr, Stalvey JR, and Warren DWd (1983). Delay in the age of balano-preputial skinfold cleavage and alterations in serum profiles of testosterone, 5 alpha-androstane-3 alpha,17 beta- diol, and gonadotropins in adult rats treated during puberty with luteinizing hormone releasing hormone. *Steroids* **41:** 443–54.

Witorsch RJ and Kitay JI (1972). Pituitary hormones affecting adrenal 5alpha-reductase activity: ACTH, growth hormone, and prolactin. *Endocrinology* **91:** 764–9.

Wray S and Hoffman G (1986). Postnatal morphological changes in rat LHRH neurons correlated with sexual maturation. *Neuroendocrinology* **43:** 93–7.

Wurtman RJ and Axelrod J (1966). The physiologic effects of melatonin and the control of its biosynthesis. *Probl Actuels Endocrinol Nutr* **10:** 189–200.

Yamashita S and Melmed S (1986). Insulin-like growth factor I action on rat anterior pituitary cells: suppression of growth hormone secretion and messenger ribonucleic acid levels. *Endocrinology* **118:** 176–82.

You L and Sar M (1998). Androgen receptor expression in the testes and epididymides of prenatal and postnatal Sprague-Dawley rats. *Endocrine* **9:** 253–61.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, and Friedman JM (1994). Positional cloning of the mouse obese gene and its human homologue [published erratum appears in *Nature* 1995 Mar 30;374(6521):479] [see comments]. *Nature* **372:** 425–32.

Zipf WB, Payne AH, and Kelch RP (1978). Prolactin, growth hormone, and luteinizing hormone in the maintenance of testicular luteinizing hormone receptors. *Endocrinology* **103:** 595–600.

Zitouni M, Pevet P, and Masson-Pevet M (1996). Brain and pituitary melatonin receptors in male rat during postnatal and pubertal development and the effect of pinealectomy and testosterone manipulation. *J Neuroendocrinol* **8:** 571–7.